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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this research is to gain an understanding of the vitamin D - androgen interaction in prostate cancer. Calcitriol, the active hormonal form of vitamin D, induces androgen receptors (AR) in prostate cells. Major findings thus far involve studies in two areas. First, the ability of vitamin D and retinoids to regulate AR levels in LNCaP human prostate cancer cells. These studies have led to a publication which is included in the Appendix. The major conclusion is that the growth inhibitory activity of vitamin D and retinoid hormones is androgen-dependent and the activity can be blocked by the AR antagonist, Casodex. Second, is a study of prostate cancer cells derived from a bone metastasis in a patient whose prostate cancer had progressed to become androgen-independent. As detailed in a paper that is in press, the cells have a double mutation in the AR. Further study of these cells is in progress in an attempt to determine the nature of the changes that led to androgen-independence.

The significance of this work is that it will increase our understanding of factors that stimulate prostate cancer growth and will attempt to develop mechanisms to inhibit the growth and progression of prostate cancer.

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FOREWORD

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David Feldman 9/1/99

PI - Signature Date

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Introduction

Calcitriol, the active hormonal form of Vitamin D, acts as a transcriptional regulator of target genes by interacting with a nuclear receptor known as the vitamin D receptor (VDR). The presence of VDR has been demonstrated in prostate cells, both primary cultures of normal prostate as well as in prostate cancer cell lines [1-4]. Calcitriol, acting via the VDR, inhibits prostate cancer cell growth as well as induces a number of target genes [4-8]. Of interest to this proposal, we have shown that calcitriol induces the androgen receptor (AR) in prostate cancer cells [9]. The basis of this grant is to investigate the role of vitamin D in prostate cancer with a focus on its role to increase AR abundance. We are pursuing several questions regarding the vitamin D - androgen interaction as they might impact on a number of aspects of prostate cancer biology: 1) androgen responsiveness, 2) effect of vitamin D on androgen ablation therapy, 3) the development of androgen-independence, and 4) the vitamin D axis as a determinant of prostate cancer risk.

Body

1. Liarozole acts synergistically with 1α,25-dihydroxy-vitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase.

Lan H. Ly, Xiao-Yan Zhao, Leah Holloway, and David Feldman

Endocrinology 140:2071-2076, 1999. This publication is in the Appendix.

1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits the proliferation of many cancer cells in culture but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-$(OH)_2D_3$-resistant phenotype in DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-$(OH)_2D_3$. Since this P450 enzyme initiates 1,25-$(OH)_2D_3$ catabolism, we presumed a high level of enzyme induction could limit the effectiveness of the 1,25-$(OH)_2D_3$ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. Since imidizole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing 1,25-$(OH)_2D_3$ half-life, thereby enhancing 1,25-$(OH)_2D_3$ antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. Neither 1,25-$(OH)_2D_3$ (1-10 nM) nor liarozole (1-10 μM) inhibited DU 145 cell growth when given alone. However, together at 4 days, 1,25-$(OH)_2D_3$ (10 nM) and liarozole (1 μM) inhibited growth 65%. We used a thin layer chromatography method to assess 24-hydroxylase activity and demonstrated that liarozole (1-100 μM) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-$(OH)_2D_3$ half-life from 11 to 31 h. In addition, 1,25-$(OH)_2D_3$ can cause homologous up-regulation of the VDR and in the presence of liarozole, this effect was

amplified thus enhancing 1,25-$(OH)_2D_3$ activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-$(OH)_2D_3$/liarozole showed greater VDR up-regulation than with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-$(OH)_2D_3$ to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity leading to increased 1,25-$(OH)_2D_3$ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-$(OH)_2D_3$ and liarozole or other inhibitors of 24-hydroxylase, both in non-toxic doses, might serve as an effective treatment for prostate cancer.

2. Induction of Androgen Receptor by 1α, 25-Dihydroxyvitamin D_3 and
9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells
by: Xiao-Yan Zhao, Lan H. Ly, Donna M. Peehl, And David Feldman
Endocrinology140: 1205-1212, 1999. This publication is included in the appendix.

The study is based on our recent findings that 1,25-$(OH)_2D_3$ inhibits proliferation of LNCaP cells, an androgen-responsive human prostate cancer cell line. Also, 1,25-$(OH)_2D_3$ increases androgen receptor (AR) abundance and enhances cellular responses to androgen in these cells. In the current study, we have investigated the mechanism by which 1,25-$(OH)_2D_3$ regulates AR gene expression and the involvement of AR in the 1,25-$(OH)_2D_3$-and 9-cis retinoic acid (RA)- mediated growth inhibition of LNCaP. Northern blot analyses demonstrated that the steady-state mRNA level of AR was significantly increased by 1,25-$(OH)_2D_3$ in a dose-dependent manner. Time course experiments revealed that the increase of AR mRNA by 1,25-$(OH)_2D_3$ exhibited delayed kinetics. In response to 1,25-$(OH)_2D_3$, AR mRNA levels were first detected to rise at 8 h and reached a maximal induction of 10-fold over the untreated control at 48 h; the effect was sustained at 72 h. Furthermore, the induction of AR mRNA by 1,25-$(OH)_2D_3$ was completely abolished by incubation of cells with cycloheximide, a protein synthesis inhibitor. 1,25-

$(OH)_2D_3$ was unable to induce expression of an AR promoter-luciferase reporter. These findings indicate that the stimulatory effect of 1,25-$(OH)_2D_3$ on AR gene expression was indirect. Western blot analyses showed an increase of AR protein in 1,25-$(OH)_2D_3$-treated cells. This increased expression of AR was followed by an inhibition of growth in LNCaP cells by 1,25-$(OH)_2D_3$. Similar to 1,25-$(OH)_2D_3$, 9-cis RA also induced AR mRNA expression and the effect of both hormones was additive. Moreover, 1,25-$(OH)_2D_3$ and 9-cis RA acted synergistically to inhibit LNCaP cell growth. These anti-proliferative effects of 1,25-$(OH)_2D_3$ and 9-cis RA alone or in combination were blocked by the pure AR antagonist, Casodex. In conclusion, our results demonstrate that growth inhibition of LNCaP by 1,25-$(OH)_2D_3$ and 9-cis RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. This finding that differentiating agents such as vitamin D and A derivatives are potent inducers of AR may have clinical implications in the treatment of prostate cancer.

3. Two Mutations Identified In The Androgen Receptor Of A New Human Prostate Cancer Cell Line MDA PCa 2a

by: Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone, Donna M. Peehl, And David Feldman

In this study, we have characterized the androgen receptor (AR) in a new human prostate cancer cell line, MDA PCa 2a, that has recently been established from a bone metastasis of a patient whose cancer was exhibiting androgen-independent growth. These cells express abundant AR (Nmax=685±149 fmol/mg protein), as determined by equilibrium binding assays with [^{3}H]dihydrotestosterone (DHT). However, Scatchard analyses show the AR binding affinity for DHT in these cells to be only 25 nM, a 50-fold lower affinity than those of the mutated AR in LNCaP cells (Kd=0.5 nM) or the wildtype AR in MCF-7 cells (Kd=0.4 nM). DNA sequence analyses of the AR gene in MDA PCa 2a cells revealed two mutations in the ligand-binding domain, L701H and T877A, the latter

being reported previously in LNCaP cells. Compared to LNCaP, the new cell line is 10 to 1000-fold less responsive to androgens in cell growth assays as well as in stimulation of PSA secretion. Interestingly, in the absence of added androgens, the new cell line expresses 15-fold higher baseline levels of PSA than LNCaP, suggesting constitutive expression of its PSA gene. In summary, we have identified two mutations in the AR gene of the MDA PCa 2a cell line that are likely responsible for decreased binding affinity for DHT and partial androgen insensitivity observed in these cells. Both androgen insensitivity and elevated baseline PSA levels exemplify the androgen-independent phenotype. Thus, this new cell line can serve as a functionally relevant model system of advanced prostate cancer and can be used to study important events related to androgen-independent growth.

Key Research Accomplishments

1. AR expression is increased in prostate cancer cells by vitamin D and retinoids.
2. The ability of vitamin D and retinoids to inhibit LNCaP cell growth is AR-dependent.
3. The MDA PCa 2A cell line has two mutations in the ligand binding domain of the AR which reduce its affinity for androgens.

Reportable Outcomes

Manuscripts

1. Liarozole acts synergistically with 1α,25-dihydroxy-vitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase

Lan H. Ly , Xiao-Yan Zhao, Leah Holloway, and David Feldman

2. Induction of Androgen Receptor by 1α, 25-Dihydroxyvitamin D_3 and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells

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Endocrinology 140:1205-1212, 1999

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by: Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone, Donna M. Peehl, And David Feldman

Journal of Urology, In press

Abstracts

1. Liarozole acts synergistically with 1α,25-dihydroxy-vitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase

Xiao-Yan Zhao, Lan H. Ly, Leah Holloway, and David Feldman

Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

Amer Soc Bone & Mineral Research, Dec 1-6, 1998 San Francisco, Pg. S265, abstract T267

2. Androgen insensitivity due to a double mutation in the androgen receptor of a new human prostate cancer cell line MDA PCa 2a Xiao-Yan Zhao[1], Bryan Boyle[1], Aruna V. Krishnan[1], Peter J. Malloy[1], Nora M. Navone[3], Donna M. Peehl[2], and David Feldman[1]. Departments of [1]Medicine and [2]Urology, Stanford University, Palo Alto, CA 94305 and [3]Department of GU Medical Oncology, M.D. Anderson Cancer Center, Houston, TX 77030

Endocrine Society 81st Annual Meeting, June 12-15, 1998, San Diego, CA. Pg 264, Abstract P1-613

Conclusions

The role of vitamin D in the prostate is complex and interesting. Vitamin D regulates AR abundance altering the cellular response to androgens. The vitamin D-androgen axis appears to be important in the progression from androgen-dependent to androgen-independent growth. Further studies are warranted to elucidate various aspects of the hormonal regulation of prostate cancer cells in the hopes of developing new therapeutic strategies to treat this common malignancy of men.

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Appendicies

1. Liarozole acts synergistically with 1α,25-dihydroxy-vitamin D3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase.

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by: Xiao-Yan Zhao, Bryan Boyle, Aruna V. Krishnan, Nora M. Navone, Donna M. Peehl, And David Feldman

81st Annual Meeting of the Endocrine Society, June 12-15, 1999.

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Liarozole Acts Synergistically with 1α,25-Dihydroxyvitamin D_3 to Inhibit Growth of DU 145 Human Prostate Cancer Cells by Blocking 24-Hydroxylase Activity*

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ABSTRACT

1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits the proliferation of many cancer cells in culture, but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-$(OH)_2D_3$-resistant phenotype in DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-$(OH)_2D_3$. As this P450 enzyme initiates 1,25-$(OH)_2D_3$ catabolism, we presumed that a high level of enzyme induction could limit the effectiveness of the 1,25-$(OH)_2D_3$ antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. As imidizole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing the 1,25-$(OH)_2D_3$ half-life, thereby enhancing 1,25-$(OH)_2D_3$ antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. When used alone, neither 1,25-$(OH)_2D_3$ (1–10 nM) nor liarozole (1–10 μM) inhibited DU 145 cell growth. However, when added together, 1,25-$(OH)_2D_3$ (10 nM)/liarozole (1 μM) inhibited growth 65% after 4 days of culture. We used a TLC method to assess 24-hydroxylase activity and demonstrated that liarozole (1–100 μM) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-$(OH)_2D_3$ half-life from 11 to 31 h. In addition, 1,25-$(OH)_2D_3$ can cause homologous up-regulation of the vitamin D receptor (VDR), and in the presence of liarozole, this effect was amplified, thus enhancing 1,25-$(OH)_2D_3$ activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-$(OH)_2D_3$/liarozole showed greater VDR up-regulation than cells treated with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-$(OH)_2D_3$ to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity, leading to increased 1,25-$(OH)_2D_3$ half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-$(OH)_2D_3$ and liarozole or other inhibitors of 24-hydroxylase, both in nontoxic doses, might serve as an effective treatment for prostate cancer. (*Endocrinology* **140:** 2071–2076, 1999)

THE MAJOR biological action of 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the active metabolite of vitamin D, is to maintain calcium homeostasis in the body (1). Recent findings indicate that 1,25-$(OH)_2D_3$ is also involved in regulating cellular proliferation and differentiation in various target tissues that possess vitamin D receptors (VDR) (1–4). 1,25-$(OH)_2D_3$ and less calcemic analogs have been shown to inhibit cell growth in both human prostate carcinoma cell lines (5–11) and primary cultures of normal and prostate cancers (12). However, 1,25-$(OH)_2D_3$ showed only minimal inhibition of cell proliferation of DU 145, a human prostate cancer cell line derived from a brain metastasis, despite the presence of substantial amounts of VDR in this cell type (5, 7). The mechanism for the relative unresponsiveness of DU 145 to the antiproliferative action of 1,25-$(OH)_2D_3$ is not known.

DU 145 cells have been shown to express high levels of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) after treatment with 1,25-$(OH)_2D_3$ (5, 7). LNCaP cells can be induced to express low levels of 24-hydroxylase activity [$12.6 \pm 3.1 \times 10^{-9}$ μmol/2 × 10^6 cells·30 min of 24,25$(OH)_2D_3$ produced] and are substantially growth inhibited by 1,25-$(OH)_2D_3$, whereas DU 145 cells can be induced to express very high levels of 24-hydroxylase activity ($96.7 \pm 39.5 \times 10^{-9}$ μmol/2 × 10^6 cells·30 min) and are minimally growth inhibited (5, 7). As this P450 enzyme initiates the 1,25-$(OH)_2D_3$ inactivation pathway (1), we (8) and others (7, 13) have considered the possibility that rapid breakdown of 1,25-$(OH)_2D_3$ by 24-hydroxylase might be the cause of the resistant phenotype in DU 145 cells. In this study, we examine the premise that combination treatment with 1,25-$(OH)_2D_3$ and an inhibitor of 24-hydroxylase might render DU 145 cells more sensitive to the antiproliferative action of 1,25-$(OH)_2D_3$.

Combination therapy is often used to enhance the anticancer activity of various agents. Ketoconazole, liarozole, and other inhibitors of P450 enzymes may exhibit anticancer properties via several pathways, including actions on critical enzyme pathways (13, 14). In this study, we examined the possibility that combination treatment with 1,25-$(OH)_2D_3$ and liarozole, an imidazole derivative with antiprostate cancer properties (15, 16), might result in enhanced growth inhibition of DU 145 cells. Liarozole is known to inhibit

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several cytochrome P-450 enzymes, including retinoic acid 4-hydroxylase and aromatase (15, 17, 18). It is suspected that the former activity prolongs the half-life of retinoic acid and thereby increases the antiproliferative activity of endogenous retinoic acid when liarozole is administered to patients with prostate cancer (15, 16, 18). Here we show that 1,25-$(OH)_2D_3$ and liarozole interact synergistically to inhibit DU 145 cell growth. Our data demonstrate, for the first time, the ability of liarozole to directly inhibit 24-hydroxylase activity. The mechanism of liarozole action on DU 145 cells appears to be via inhibition of 24-hydroxylase, which causes a dual effect to prolong 1,25-$(OH)_2D_3$ half-life and to enhance up-regulation of VDR levels. Additional mechanisms may also play a role.

Materials and Methods

Materials

25-Hydroxy-[^{3}H]vitamin D_3 (SA, 12.7 Ci/mmol) was obtained from Amersham Chemical Co. (Arlington Heights, IL). Liarozole fumarate (5-[(3-chlorophenyl)(1*H*-imidazol-1-yl)methyl]1*H*-benzimidazole fumarate) was a gift from Dr. C. Bowden (Janssen Research Foundation, Spring House, PA), and 1,25-$(OH)_2D_3$ was a gift from Dr. M. Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture media were purchased from Mediatech (Herndon, VA). FBS was obtained from Life Technologies (Gaithersburg, MD). CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS reagent) was purchased from Promega Corp. (Madison, WI). Silica gel TLC plates were purchased from E. M. Science (Darmstadt, Germany). All other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

The DU 145 human prostate carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely cultured in RPMI 1640 medium supplemented with 5% FBS and antibiotics at 37 C in a humidified atmosphere of 5% CO_2.

Assay of cell growth

Cell growth was assessed by measurement of viable cells using the MTS assay. DU 145 cells were trypsinized and seeded at a density of approximately 2,000 cells/well in 96-well tissue culture plates (Falcon, Lincoln Park, NJ) in 200 μl culture medium. The cells were allowed to attach for 24 h, and the medium was replaced with fresh medium containing 5% FBS. Cells were then treated with vehicle (ethanol), 1,25-$(OH)_2D_3$, and/or liarozole. Triplicate wells were used for each experimental condition. The medium containing vehicle or test compounds was renewed every 2 days during the course of the experiment. After the appropriate incubation period, the cells were processed by replacing them with fresh RPMI 1640 medium containing MTS reagent (100 μl medium plus 20 μl MTS reagent/well). The plates were incubated at 37 C in a humidified atmosphere of 5% CO_2 for approximately 3–4 h. The absorbance at 490 nm was read using an automatic plate reader (Emax Precision Microplate Reader, Molecular Devices, Menlo Park, CA) and was linear up to the highest cell concentration tested (40,000 cells/well).

Induction of 24-hydroxylase activity

24-Hydroxylase enzyme activity was assayed in a cell suspension system slightly modified from the method previously described (19). A 100-mm^2 confluent DU 145 culture, growing under standard conditions, was treated for various times (0.5, 3, 6, 16, and 20 h) with either vehicle (ethanol) or 10 nM 1,25-$(OH)_2D_3$. Cells were then rinsed with 10 ml PBS and incubated with 10 ml culture medium at 37 C in a humidified atmosphere of 5% CO_2 for approximately 30 min to remove 1,25-$(OH)_2D_3$. Cells were then trypsinized and resuspended at 10^6 cells/200 μl RPMI 1640 containing 10 mM HEPES with 1% FBS. The cells were incubated for 30 min at 37 C with 1.0 nM [^{3}H]25-OHD_3 and 1.0 μM 25-$(OH)D_3$. The reaction was terminated by the addition of 750 μl methanol-chloroform (2:1) and 20 μl 24,25-$(OH)_2D_3$. The metabolites were extracted three times with 200 μl chloroform. The organic extracts were combined, dried with a Speed-Vac (Savant Instruments, Farmingdale, NY) and dissolved in a 90:10 mixture of hexane-isopropanol. The production of [^{3}H]24,25-$(OH)_2D_3$ was quantitated by TLC on silica gel/aluminum foil plates developed in methylene chloride-ethyl acetate (1:1) run with authentic standards. The TLC strips were cut into 14 fractions and placed individually in minicounting vials. This TLC system produced good separation of [^{3}H]25-$(OH)_2D_3$ from [^{3}H]24,25-$(OH)_2D_3$.

Inhibition of 24-hydroxylase activity

Time-course studies indicate that induction of 24-hydroxylase activity could be detected at 3 h by 10 nM 1,25-$(OH)_2D_3$ treatment with a plateau at approximately 20 h. Therefore, the conditions selected for studying the inhibition of 24-hydroxylase activity by liarozole were 20-h induction, 10^6 cells, 1 nM [^{3}H]25-$(OH)D_3$, and 30-min incubation with various concentrations of liarozole (1, 10, 50, and 100 μM).

Determination of 1,25-$(OH)_2D_3$ half-life

The half-life of 1,25-$(OH)_2D_3$ in DU 145 cells was determined by measuring the residual unmetabolized [^{3}H]1,25-$(OH)_2D_3$ in the conditioned medium at various time points after addition. Confluent DU 145 cells were treated for various times with [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of liarozole. Two hundred microliters of conditioned medium were mixed with 750 μl methanol-chloroform (2:1), and the metabolites were extracted with 200 μl chloroform. Chloroform extraction was repeated three times. The organic extracts were dried with a Speed-Vac and dissolved in a 90:10 mixture of hexane-isopropanol. The disappearance of [^{3}H]1,25-$(OH)_2D_3$ and the production of [^{3}H]1,24,25-$(OH)_3D_3$ were quantitated by a TLC system using methylene chloride-ethyl acetate (1:3). After 145 min of development, the TLC strips were dried and fractionated by cutting regions identified as 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ by comigration of authentic standards. This TLC system gave good separation between [^{3}H]1,25-$(OH)_2D_3$ and [^{3}H]1,24,25-$(OH)_3D_3$. The Rf value for 1,25-$(OH)_2D_3$ was 0.667, and that for 1,24,25-$(OH)_3D_3$ was 0.333.

Western blot analysis of vitamin D receptor (VDR)

Cell monolayers grown in RPMI 1640 supplemented with 5% charcoal-stripped serum in 100-mm dishes were incubated with ethanol vehicle, 1,25-$(OH)_2D_3$ (0.1, 1, and 10 nM), and/or liarozole (10 μM) for 4 days. After 4 days of incubation, cells were harvested, and Western blot analysis was performed as described previously using anti-VDR monoclonal antibody (9A7) (20). The experiment was repeated twice with similar results.

Results

Combination effect of 1,25-$(OH)_2D_3$ and liarozole on DU 145 cell growth

DU 145 cells are only minimally responsive to the antiproliferative effect of 1,25-$(OH)_2D_3$ (5, 7, 8). In our current studies, DU 145 cells were treated with increasing concentrations of 1,25-$(OH)_2D_3$ (1, 10, and 100 nM) over a time course of 2, 4, and 6 days (Fig. 1, A and B). The growth of DU 145 cells was not significantly inhibited by the lower concentrations of 1 and 10 nM; however, at the highest concentration (100 nM), there was a slight growth inhibition of approximately 20% on day 6. Similarly, as shown in Fig. 1, C and D, liarozole failed to inhibit the proliferation of DU 145 cells at 1 and 10 μM, but 100 μM resulted in 50% growth inhibition at 4 days and 60% growth inhibition at 6 days. However, 100 μM liarozole is a toxic dose and when administered to patients at these concentrations it causes hypervi-

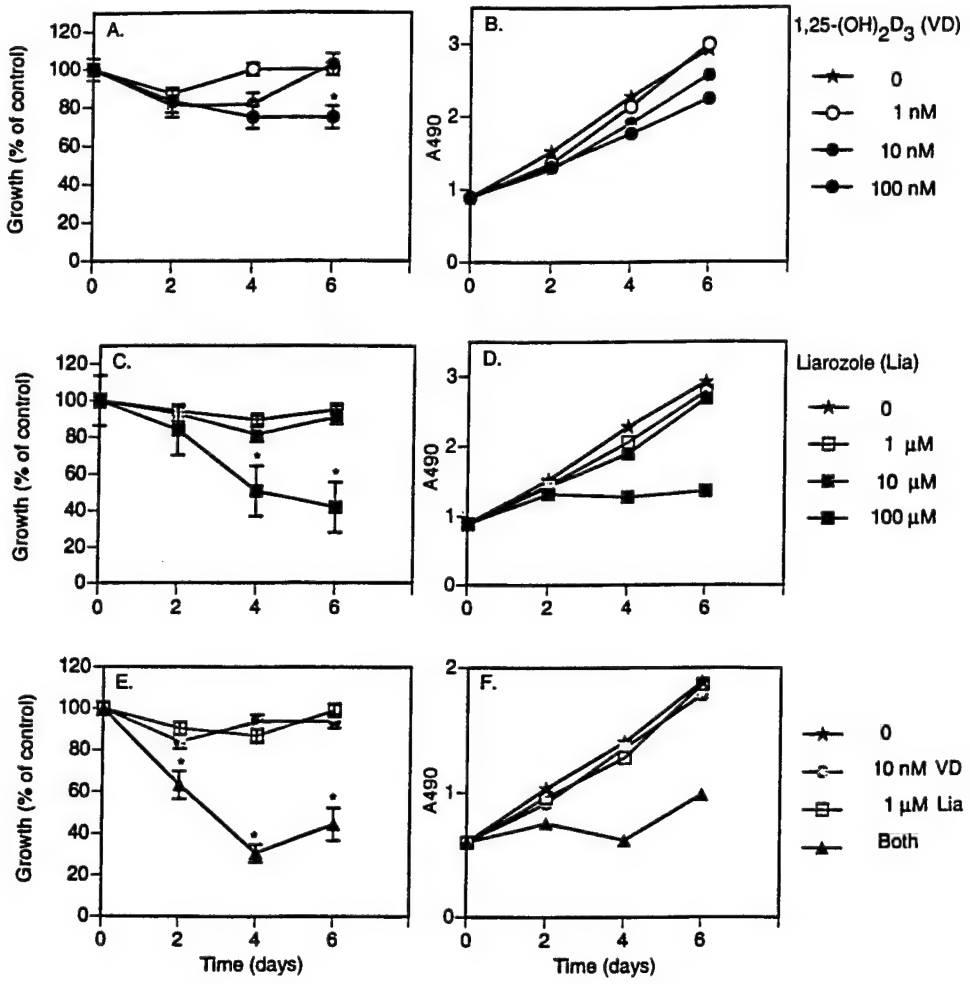

FIG. 1. Dose-response effect of 1,25-$(OH)_2D_3$, liarozole, and the combination on DU 145 cell growth over a time course of 6 days. Cells were plated at approximately 2000 cells/well in 96-well tissue culture plates in 200 μl medium with the indicated concentrations of hormone. Media were changed every 2 days. Cell proliferation was estimated using the MTS assay. Data are expressed as the mean ± SD (n = 3) in the *left panels*. The *right panels* show a single representative experiment comparing treatment to vehicle and expressed in absorbance units. *, Significant changes ($P < 0.05$) compared with the ethanol control. A and B, Treatment with 1,25-$(OH)_2D_3$. C and D, Treatment with liarozole. E and F, Treatment with a combination of 1,25-$(OH)_2D_3$ (10 nM) and liarozole (1 μM).

taminosis A. Neither 10 nM 1,25-$(OH)_2D_3$ nor 1 μM liarozole had any antiproliferative effect when used alone. However as shown in Fig. 1, E and F, the combination treatment caused 60% growth inhibition. These data indicate that 1,25-$(OH)_2D_3$ and liarozole interact synergistically to inhibit DU 145 cell growth.

Inhibition of 24-hydroxylase activity by liarozole

We next investigated the possible mechanisms by which liarozole enhanced the ability of 1,25-$(OH)_2D_3$ to inhibit DU 145 cell proliferation. As shown in many other cell culture systems, we found here that 1,25-$(OH)_2D_3$ induced 24-hydroxylase activity in DU 145 cells in a time-dependent manner (Fig. 2). The level of 24-hydroxylase activity in DU 145 cells is much higher than that in other cell types, particularly compared with LNCaP cells, which are substantially inhibited by 1,25-$(OH)_2D_3$ alone (5, 7). Liarozole had no intrinsic ability to induce 24-hydroxylase activity. However, liarozole can inhibit 24-hydroxylase activity. After treating cells with 10 nM 1,25-$(OH)_2D_3$ for 20 h to induce 24-hydroxylase activity, we examined the abilities of various concentrations of liarozole to inhibit enzyme activity by blocking the conversion of [^{3}H]25-OHD_3 to 24,25-$(OH)_2D_3$. As shown in Fig. 3, liarozole (1, 10, 50, and 100 μM) was able to directly inhibit 24-hydroxylase activity in a dose-dependent manner, such that 10 μM liarozole resulted in approximately 80% inhibition of enzyme activity compared with the activity of the induced cells in the absence of liarozole.

Effect of liarozole on 1,25-$(OH)_2D_3$ half-life

As our data indicated that liarozole was capable of directly inhibiting 24-hydroxylase activity, the enzyme involved in the first step of 1,25-$(OH)_2D_3$ inactivation, we next investigated the effect of liarozole on the 1,25-$(OH)_2D_3$ half-life. We treated two group of cells at various times, one with the single addition of 10 nM 1,25-$(OH)_2D_3$ and the other with a combination of 10 nM 1,25-$(OH)_2D_3$ and 1 μM liarozole. As anticipated, in cultures treated with 1,25-$(OH)_2D_3$ alone, the half-life of 1,25-$(OH)_2D_3$ was shorter (~10 h) compared with that of cells treated with the combination (~30 h; Fig. 4). Therefore, these data are consistent with the observation that liarozole directly inhibits 24-hydroxylase activity, thereby prolonging 1,25-$(OH)_2D_3$ half-life. This finding provides one possible mechanism for the synergistic growth inhibitory effect of combination therapy with 1,25-$(OH)_2D_3$ and liarozole.

Effect of 1,25-$(OH)_2D_3$ and liarozole on VDR protein level

As previously shown in other cell culture systems, 1,25-$(OH)_2D_3$ and other vitamin D analogs induce homologous

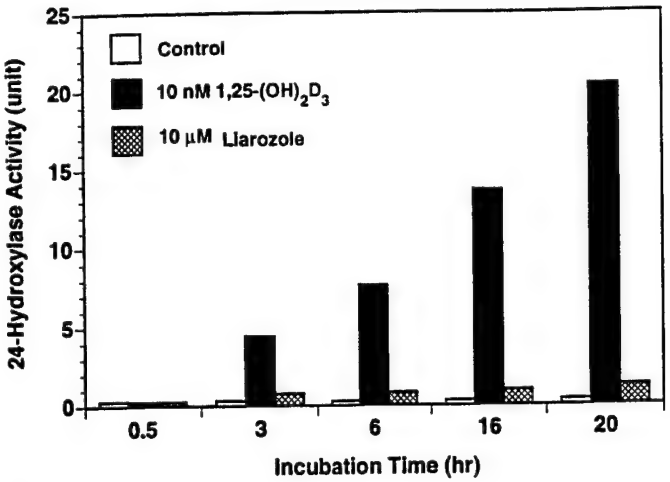

FIG. 2. Time course of the effect of 1,25-$(OH)_2D_3$ or liarozole on 24-hydroxylase activity in DU 145 cells. Cells were treated with 10 nM 1,25-$(OH)_2D_3$, 10 μM liarozole, or ethanol vehicle, and enzyme activity was measured at 0.5, 3, 6, 16, and 20 h. At 20 h, 10 nM 1,25-$(OH)_2D_3$ induced a 27-fold rise in 24-hydroxylase activity compared with the effect of vehicle. This is a representative experiment that was performed twice with similar results.

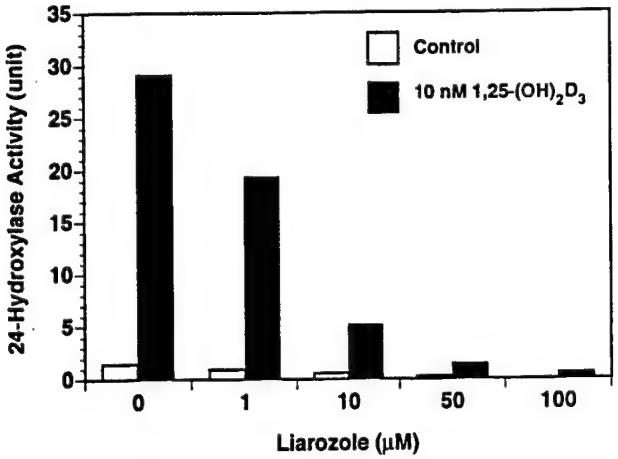

FIG. 3. Dose-dependent effect of liarozole on 24-hydroxylase activity in DU 145 cells. Cells were treated with 10 nM 1,25-$(OH)_2D_3$ for 20 h. Treated cells were subsequently incubated with liarozole at various concentrations (0, 1, 10, 50, and 100 μM) for 30 min before enzyme activity was measured. This is a representative experiment that was performed three times with similar results.

up-regulation of the VDR (21, 22). We next investigated whether liarozole, by inhibiting 24-hydroxylase activity and prolonging 1,25-$(OH)_2D_3$ half-life, is also capable of augmenting VDR up-regulation. Cells were treated with various concentrations of 1,25-$(OH)_2D_3$ (0.1, 1, and 10 nM) with and without the addition of 10 μM liarozole over a time course of 4 days. Protein extracts were made from these treated cells and were subjected to Western blot analysis to evaluate VDR content. Using the monoclonal antibody 9A7, the 50-kDa VDR protein was visualized. As shown in Fig. 5A, 1,25-$(OH)_2D_3$ alone resulted in a slight up-regulation of the VDR protein level (2-fold). Liarozole alone had no significant effect on VDR abundance. However, the combination of 1,25-$(OH)_2D_3$ and liarozole resulted in a 1,25-$(OH)_2D_3$ dose-dependent increase in the VDR protein level. This effect was

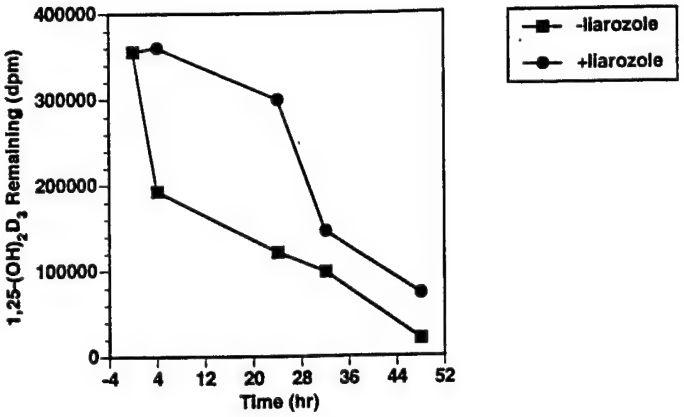

FIG. 4. Effect of liarozole on 1,25-$(OH)_2D_3$ half-life. DU 145 cells were incubated with [^{3}H]1,25-$(OH)_2D_3$ (0.5 nM) plus unlabeled 1,25-$(OH)_2D_3$ (10 nM) in the absence or presence of 10 μM liarozole. Conditioned media were collected at various time points (0, 4, 24, 32, and 48 h), and the residual amount of unmetabolized [^{3}H]1,25-$(OH)_2D_3$ was determined by TLC. This is a representative experiment performed twice with similar results.

observed most profoundly after a combination treatment with 10 nM 1,25-$(OH)_2D_3$ and 10 μM liarozole. This combination, as shown in Fig. 5B, resulted in a 5-fold increase in VDR abundance. Our data suggest that an increase in the VDR protein level may serve as a second and related mechanism, added to the prolonged 1,25-$(OH)_2D_3$ half-life, that contributes to the synergistic effect of 1,25-$(OH)_2D_3$ and liarozole inhibition of DU 145 cell growth.

Discussion

Our study was designed to investigate the combination of 1,25-$(OH)_2D_3$ and liarozole as a possible treatment for prostate cancer. We carried out our studies using an aggressive human prostate cancer cell line, DU 145, because of its resistance to the growth inhibitory effects of 1,25-$(OH)_2D_3$. We successfully inhibited DU 145 cell growth by 60% using the combination treatment of 1,25-$(OH)_2D_3$ and liarozole. In addition, we gained insight into the mechanism of the DU 145 cell unresponsiveness and the possibility of reversing the resistance with combination therapy.

As discussed earlier, at concentrations that are nontoxic, neither liarozole nor 1,25-$(OH)_2D_3$ alone substantially inhibited cell growth. Yet the combination of 1 μM liarozole and 10 nM 1,25-$(OH)_2D_3$ resulted in significant synergistic antiproliferative effects. Furthermore, this synergy is observed at a pharmacologically relevant concentration for both compounds. Based on this observation, we explored the possible mechanisms behind this synergy. We discovered that liarozole directly inhibited 24-hydroxylase activity in addition to inhibiting the already known P-450 enzymes, such as 4-hydroxylase and aromatase (15, 17, 18). As 24-hydroxylase is the initial enzyme for inactivating 1,25-$(OH)_2D_3$, we measured 1,25-$(OH)_2D_3$ half-life, and indeed, it was prolonged from 11 to 31 h. Therefore, by preventing rapid inactivation of 1,25-$(OH)_2D_3$ and prolonging the exposure time of cells to active hormone, DU 145 cells were able to respond to its antiproliferative effect. This provided the first possible mechanism for the synergistic activity of the 1,25-$(OH)_2D_3$/liarozole combination.

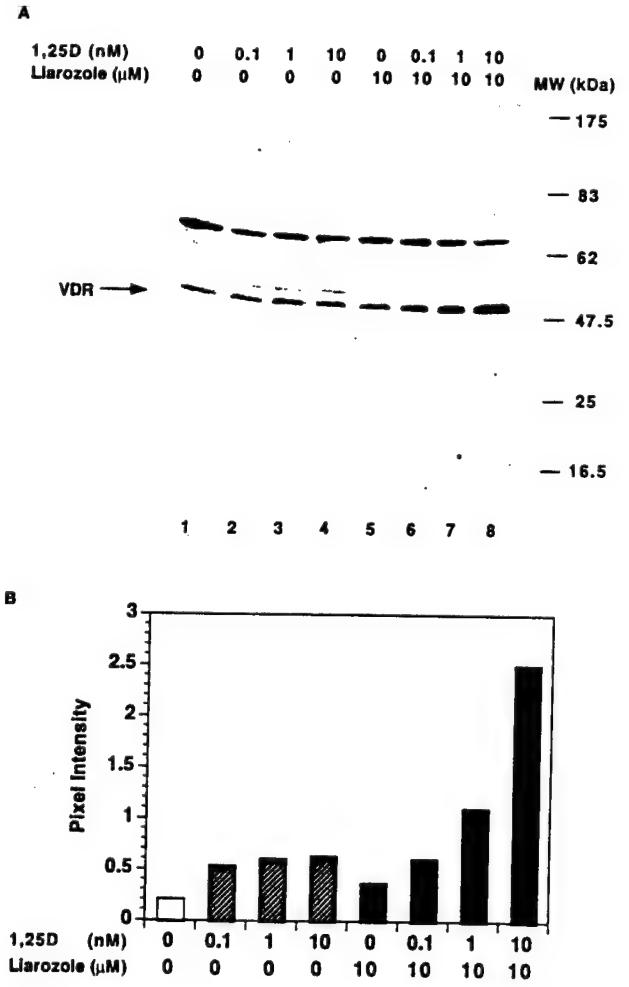

FIG. 5. A, Western blot analysis of VDR levels in DU 145 cells in response to various treatments. Cells were treated with ethanol or increasing concentrations of 1,25-$(OH)_2D_3$, liarozole, or 1,25-$(OH)_2D_3$ and liarozole for 4 days. High salt extracts were prepared, and 100 μg protein were loaded onto an 8% SDS-PAGE. After gel transfer, the blot was probed with anti-VDR monoclonal antibody 9A7, and the signal was detected using the enhanced chemiluminescence method. B, Densitometric analysis of Western blot. The pixel intensities of VDR bands were quantitated using a laser densitometer.

It is known that receptor regulation is an important mechanism for modulating target cell responsiveness to hormone (20, 23, 24). We explored the possibility that homologous up-regulation of the VDR would be enhanced in the presence of liarozole. Western blot analysis demonstrated a 5-fold increase in VDR protein level when cells were treated with 10 nM 1,25-$(OH)_2D_3$ and 10 μM liarozole compared with that after treatment with 1,25-$(OH)_2D_3$ alone. DU 145 cells treated with 1,25-$(OH)_2D_3$ alone only demonstrated a slight up-regulation of the VDR protein level. The possible explanation for this observation is that 1,25-$(OH)_2D_3$ is a potent stimulus of 24-hydroxylase activity; therefore, this would induce rapid degradation of 1,25-$(OH)_2D_3$, causing only a transient homologous up-regulation of VDR in DU 145 cells. Augmentation of VDR up-regulation has previously been reported using ketoconazole to inhibit 24-hydroxylase in a similar manner (25, 26). Enhanced VDR up-regulation is the second contributing mechanism explaining the liarozole synergistic interaction with 1,25-$(OH)_2D_3$. The increase in both ligand and receptor is a plausible mechanism for the enhanced antiproliferative activity of the 1,25-$(OH)_2D_3$/liarozole combination therapy in DU 145 cells. It is of interest that analogs of 1,25-$(OH)_2D_3$ designed to prevent 24-hydroxylation, such as 19-nor-25,26-hexafluoro-1,25-$(OH)_2D_3$, inhibit DU145 cell proliferation (9).

Our data suggest that DU 145 cells are more responsive to the antiproliferative effect of 1,25-$(OH)_2D_3$ when both its hormone and receptor are increased. Although the presence of VDR is essential for 1,25-$(OH)_2D_3$ activity (27), the level of VDR abundance in different prostate cancer cell lines by itself is not necessarily predictive of the amplitude of hormonal response (5, 28). However, in a given cell, increased abundance of receptor does appear to predict the extent of hormonal responsiveness, and increased or decreased receptor levels are usually correlated with increased and decreased responsiveness, respectively (20, 23, 24).

It should be noted that Zhao *et al.* have shown that combination therapy with either ketoconazole or liarozole and 1,25-$(OH)_2D_3$ or its analogs is cell type specific (13). That finding supports the concept that differences in cellular metabolism can at least partially explain the different potencies of various vitamin D analogs and differences in antiproliferative activity between different cancer cells. The fact that some cells are substantially growth inhibited by 1,25-$(OH)_2D_3$ alone (LNCaP and primary cultures) and other cells are not (DU 145) depends on a combination of factors, including, but not limited to, VDR abundance and inducible 24-hydroxylase activity (5, 7, 28). Liarozole in combination with 1,25-$(OH)_2D_3$ improves both parameters; by increasing VDR abundance and inhibiting 24-hydroxylase activity, it allows the otherwise resistant DU 145 cell to be growth arrested by 1,25-$(OH)_2D_3$. In preliminary experiments, liarozole also augmented the growth inhibitory activity of 1,25-$(OH)_2D_3$ in PC-3 and LNCaP cells, but to a much lesser extent (data not shown) than shown here for DU145 cells. The smaller augmentation was probably due to the greater antiproliferative activity of 1,25-$(OH)_2D_3$ alone in these cells (5) as well as the lesser induction of 24-hydroxylase in these cell lines (7), making the liarozole action to inhibit 24-hydroxylase less essential for 1,25-$(OH)_2D_3$-mediated growth inhibition.

Another possible mechanism for the enhanced antiproliferative effect in the presence of liarozole is its ability to inhibit retinoid metabolism, leading to increased retinoid levels (15, 18, 29). Retinoids have been shown to inhibit various cancer cell lines, including prostate cancer (30), and to be synergistic with 1,25-$(OH)_2D_3$ in inhibiting prostate cancer cell growth (10, 31). In fact, the beneficial effect of liarozole in patients with prostate cancer is attributed to this activity (15, 18, 29). We investigated the possibility that this activity might be contributing to the growth inhibition in our experiments. We treated DU 145 cells with a combination of 1,25-$(OH)_2D_3$ and increasing concentrations of retinoic acid to mimic the liarozole effect. We observed only a slight enhancement of growth inhibition (data not shown). As liarozole alone had no antiproliferative activity, and retinoids were not added in our standard combination experiments, we believe that the inhibition of retinoid metabolism does not substantially contribute to the effects that we have seen in cultured cells.

However, in patients, the ability of liarozole to inhibit retinoid metabolism would be expected to further enhance the synergistic activity that we have demonstrated.

The mechanism(s) by which 1,25-$(OH)_2D_3$ inhibits the growth of prostate cancer cells is complex, multifactorial, and different in different cell lines. Several investigators have reported that treatment with 1,25-$(OH)_2D_3$ causes LNCaP cells to accumulate in the G_1 phase of the cell cycle (10, 11). 1,25-$(OH)_2D_3$ also elicits a reduction of cyclin-dependent kinase 2 activity and an increase in the level of hypophosphorylated retinoblastoma (Rb) protein, which is a critical regulator of the G_1/S checkpoint (11). Interestingly, DU 145 cells lack functional Rb protein. However, ectopic expression of functional Rb in DU 145 cells was not sufficient to restore the growth response to 1,25-$(OH)_2D_3$ (32). Others have found that growth inhibition of prostate cancer cells by a potent vitamin D analog involves the induction of $p21^{waf1}$, $p27^{kip1}$, and E-cadherin (9). In addition, we have demonstrated that 1,25-$(OH)_2D_3$ significantly regulates androgen receptor gene expression, which contributes to the regulation of LNCaP cell growth (33). Therefore, the mechanism by which 1,25-$(OH)_2D_3$ inhibits cell proliferation involves multiple signaling pathways and differs in various prostate cancer cell lines.

In summary, our data suggest that liarozole directly inhibits 24-hydroxylase activity, thereby effectively prolonging the 1,25-$(OH)_2D_3$ half-life. The increase in the 1,25-$(OH)_2D_3$ half-life resulted in enhanced up-regulation of VDR protein levels. We believe that this combination of increased 1,25-$(OH)_2D_3$ hormone levels as well as augmented VDR abundance represents the principal mechanism for the synergistic effect of 1,25-$(OH)_2D_3$ and liarozole in our experiments. However, additional mechanisms may play a role in the synergistic effect of this combination in DU 145 cells. In conclusion, the novel combination of liarozole and 1,25-$(OH)_2D_3$ therapy may serve as an effective treatment regimen for prostate cancer patients.

Acknowledgments

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Induction of Androgen Receptor by 1α,25-Dihydroxyvitamin D_3 and 9-cis Retinoic Acid in LNCaP Human Prostate Cancer Cells*

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ABSTRACT

We have recently shown that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] inhibits proliferation of LNCaP cells, an androgen-responsive human prostate cancer cell line. Also, 1,25-$(OH)_2D_3$ increases androgen receptor (AR) abundance and enhances cellular responses to androgen in these cells. In the current study, we have investigated the mechanism by which 1,25-$(OH)_2D_3$ regulates AR gene expression and the involvement of AR in the 1,25-$(OH)_2D_3$- and 9-cis retinoic acid (RA)-mediated growth inhibition of LNCaP cells. Northern blot analyses demonstrated that the steady-state messenger RNA (mRNA) level of AR was significantly increased by 1,25-$(OH)_2D_3$ in a dose-dependent manner. Time-course experiments revealed that the increase of AR mRNA by 1,25-$(OH)_2D_3$ exhibited delayed kinetics. In response to 1,25-$(OH)_2D_3$, AR mRNA levels were first detected to rise at 8 h and reached a maximal induction of 10-fold over the untreated control at 48 h; the effect was sustained at 72 h. Furthermore, the induction of AR mRNA by 1,25-$(OH)_2D_3$ was completely abolished by incubation of cells with cycloheximide, a protein synthesis inhibitor. 1,25-$(OH)_2D_3$ was unable to induce expression of an AR promoter-luciferase reporter. Together, these findings indicate that the stimulatory effect of 1,25-$(OH)_2D_3$ on AR gene expression is indirect. Western blot analyses showed an increase of AR protein in 1,25-$(OH)_2D_3$-treated cells. This increased expression of AR was followed by 1,25-$(OH)_2D_3$-induced inhibition of growth in LNCaP cells. Similar to 1,25-$(OH)_2D_3$, 9-cis RA also induced AR mRNA expression, and the effect of both hormones was additive. Moreover, 1,25-$(OH)_2D_3$ and 9-cis RA acted synergistically to inhibit LNCaP cell growth. These antiproliferative effects of 1,25-$(OH)_2D_3$ and 9-cis RA, alone or in combination, were blocked by the pure AR antagonist, Casodex. In conclusion, our results demonstrate that growth inhibition of LNCaP cells by 1,25-$(OH)_2D_3$ and 9-cis RA is mediated by an AR-dependent mechanism and preceded by the induction of AR gene expression. This finding, that differentiating agents such as vitamin D and A derivatives are potent inducers of AR, may have clinical implications in the treatment of prostate cancer. (*Endocrinology* **140:** 1205–1212, 1999)

1α,25-DIHYDROXYVITAMIN D_3 [1,25-$(OH)_2D_3$], the active metabolite of vitamin D, regulates calcium homeostasis in the body by actions in the intestine, bone, kidney, and parathyroid glands (1, 2). Recently, 1,25-$(OH)_2D_3$ has also been shown to have nonclassical actions. For example, the hormone exerts antiproliferative and prodifferentiating effects on many cell types, including cells derived from myeloid, breast, colon, and prostate tissues (3–6). Biologic responses of target cells to 1,25-$(OH)_2D_3$ are mediated by its nuclear receptor, the vitamin D receptor (VDR) (7). The VDR belongs to the steroid/thyroid/retinoid receptor superfamily (1, 2). Numerous studies indicate that VDR controls target gene transcription by forming a heterodimeric complex with the retinoid X receptor (RXR), the receptor for 9-cis retinoic acid (RA), and binding to the vitamin D response element (VDRE) present in the promoter region of target genes.

Our group (8, 9), as well as others (10), have shown that VDRs are present in established human prostate cancer cell lines, as well as primary cultures of normal prostate and cancer cells (11). Moreover, 1,25-$(OH)_2D_3$ and its analogs significantly inhibit cellular proliferation of prostate cancer cells, including LNCaP (8, 9, 12–20). LNCaP cells express both the VDR and the androgen receptor (AR). Our recent studies (21) and those of others (15, 22) have demonstrated that cross-talk between 1,25-$(OH)_2D_3$ and androgens exists and that the antiproliferative actions of 1,25-$(OH)_2D_3$ in LNCaP cells are androgen-dependent. Blutt *et al.* (17) have shown that 9-cis RA acts synergistically with 1,25-$(OH)_2D_3$ to inhibit LNCaP cell growth.

Because cellular responsiveness to androgen depends on AR abundance, in the present study, we have analyzed the ability of 1,25-$(OH)_2D_3$ and 9-cis RA to regulate the level of AR gene expression in these cells. We found that 1,25-$(OH)_2D_3$ increased the levels of AR messenger RNA (mRNA) and AR protein in a concentration- and time-dependent manner. Such regulatory effects of 1,25-$(OH)_2D_3$ on AR gene expression required *de novo* protein synthesis. Furthermore, the stimulatory effect of 1,25-$(OH)_2D_3$ on AR mRNA was also enhanced by 9-cis RA. Because it has been reported that the antiproliferative effects of 1,25-$(OH)_2D_3$ on LNCaP cells can be synergistically enhanced by the addition of 9-cis RA (17), we examined the involvement of AR in the antiproliferative action of 9-cis RA, as well as 1,25-$(OH)_2D_3$. Using the pure AR antagonist, Casodex, we demonstrated that AR blockade prevented the growth inhibitory activity of both 1,25-$(OH)_2D_3$ and 9-cis RA. In contrast, Casodex did not affect the antiproliferative activity of dibutyrl cAMP, a well-known

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up-regulator of AR in LNCaP cells (23). Our studies demonstrate that both 1,25-$(OH)_2D_3$ and 9-cis RA up-regulate AR mRNA levels in LNCaP cells and that growth inhibition mediated by 1,25-$(OH)_2D_3$ and 9-cis RA requires the action of AR.

Materials and Methods

Materials

1,25-$(OH)_2D_3$ was the generous gift of Dr. M. Uskokovic (Hoffmann-LaRoche, Inc., Nutley, NJ). Bicalutamide (Casodex or ICI 17,334) was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Aprotinin, pepstatin, and soybean trypsin inhibitor were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tissue culture media were purchased from Mediatech (Herndon, VA). All other reagents, except where indicated, were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-AR monoclonal antibody F39.4 and the human AR complementary DNA (cDNA) were generous gifts from Dr. TH Van der Kwast (Erasmus University, Rotterdam, Netherlands) and Dr. M. McPhaul (University of Texas Southwestern Medical Center, Dallas, TX), respectively. FBS was obtained from Gibco BRL (Gaithersburg, MD). Charcoal-stripped FBS (CSS) was purchased from Sigma Chemical Co.

Cell culture and hormone treatment

The LNCaP human prostate carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI-1640 medium supplemented with 5% FBS and antibiotics (FBS medium), at 37 C in a humidified atmosphere of 5% CO_2. For experiments, LNCaP cells were trypsinized and seeded at an appropriate density, and hormonal treatments were initiated, the next day, in FBS medium or in RPMI-1640 medium supplemented with 5% CSS and antibiotics (CSS medium).

Hormone stocks [1,25-$(OH)_2D_3$, 9-cis RA, and Casodex] were prepared in 100% ethanol, at a concentration 1000-fold higher than the working concentrations. Fresh culture media were premixed with hormone stock and then added to triplicate wells. Media and hormone were replenished every 2 days. Controls received ethanol vehicle at a concentration equal to that in hormone-treated cells.

Assay of cell proliferation

Cell proliferation was assessed by measurement of attained cell mass using an assay of DNA content. As previously described (21), LNCaP cells were seeded in six-well tissue culture plates (Becton Dickinson and Co., Lincoln Park, NJ), at a density of 50,000 cells per well, in 3 ml RPMI-1640 containing 5% FBS. After incubation for 24 h, the medium was replaced with fresh medium containing 5% FBS (FBS medium). Cells were treated with vehicle (ethanol, final concentration 0.1%), 1,25-$(OH)_2D_3$, 9-cis RA, dibutyrl cAMP, or Casodex. On the sixth day, cell monolayers were processed for DNA assay using the method of Burton (24). DNA content of each treatment was derived from the mean value of triplicate wells in an experiment. Each experiment was repeated three times.

Western blot analysis

Cells were treated with ethanol or 1,25-$(OH)_2D_3$ (10 nM) in RPMI-1640 medium containing 5% CSS (CSS medium) for 2 days. They were harvested, and sonicated extracts were prepared as described. Aliquots of 100 μg protein were heated in SDS sample buffer at 95 C, for 5 min, before electrophoresis in an 8% SDS-polyacrylamide gel. After electrophoresis, the gels were transferred and processed as previously described (25). After transfer, the blots were incubated with anti-AR monoclonal antibody F39.4 (1:100 dilution) for 1 h, at room temperature, with gentle shaking. The blots were washed and then incubated with a horseradish peroxidase-conjugated rabbit antimouse IgG (1:1000 dilution) for 1 h at room temperature. Blots were rewashed and developed with the Enhanced Chemiluminesence (ECL) System system, according to the manufacturer's instructions (Amersham Chemical Co.).

Steroid receptor ligand-binding assay

LNCaP cells were seeded at a density of 150,000 cells per 100-mm dish in 10-ml medium containing 5% FBS or 5% CSS. At the end of the 6-day incubation with hormone (at concentrations of 0, 1, and 10 nM), cell monolayers were harvested, and high-salt nuclear extracts were made as previously described (21). Protein concentration of the extract was determined (26). In a typical binding experiment, 200 μl soluble extract (1–2 mg protein/ml) were incubated with 10 nM concentration of [^{3}H]-5α-dihydrotestosterone (DHT) for 16–20 h at 4 C. Bound and free hormone were separated by hydroxylapatite (21). Specific binding was calculated by subtracting nonspecific binding (obtained in the presence of a 250-fold excess of radioinert DHT) from the total binding (measured in the absence of radioinert steroid). Data were expressed as femtomoles [^{3}H]-DHT bound per milligram protein.

Northern blot analysis

Northern blot analysis was performed as previously described (8, 11). Briefly, semiconfluent LNCaP cells were treated with graded concentrations of 1,25-$(OH)_2D_3$, or 5 mM dibutyrl cAMP, or 9-cis RA in FBS medium and in CSS medium for 24 h before isolation of total RNA. Ten micrograms of total RNA were denatured, fractionated by electrophoresis, and transferred to Hybond-N nylon membrane (Amersham), as previously described (8, 11). The bound RNA was immobilized by UV cross-linking and then hybridized with a random primed [^{32}P]-labeled 0.8-kb *Hin*dIII-*Bam*HI fragment of the human AR cDNA at 60 C. To control for RNA sample loading and transfer, Northern blots were also hybridized with a [^{32}P]-labeled 0.9-kb *Eco*RI fragment of the human cDNA for the ribosomal protein gene L7 (8, 11). The silver grain pixel intensity of each AR and L7 band was scanned by a densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

AR promoter-luciferase reporter gene assay

LNCaP cells were seeded at 3×10^6 cells/dish in 60-mm tissue culture dishes (Corning, Inc., Corning, NY) in RPMI-1640 medium containing 5% FCS and antibiotics. A 6-kb promoter-luciferase reporter was transfected using a calcium-phosphate method (23). Each transfection contained 1 μg pAR-LUC DNA (Drs. G. Mora and D. Tindall, personal communication) and 0.1 μg pSV-Renilla DNA. The control plasmid pSV-Renilla was used to monitor transfection efficiency. Cells were harvested after 32 h of incubation with tested compounds at 37 C. Luciferase activity was employed to measure induction using Promega Corp. (Madison, WI) dual luciferase assay system on luminometer TD-20 (Turner Design, Sunnyvale, CA). The results were expressed as the ratio of luciferase activity to Renilla activity.

Statistical analysis

ANOVA was used to assess the statistical significance of the difference. $P < 0.05$ was considered significant.

Results

We have recently demonstrated that the antiproliferative action of 1,25-$(OH)_2D_3$ in LNCaP cells is androgen-dependent (21). Here, we investigate further the interaction between 1,25-$(OH)_2D_3$ and androgen signaling pathways by exploring the mechanism of 1,25-$(OH)_2D_3$ regulation of AR gene expression in these cells. We also examine the possible involvement of AR in the synergistic antiproliferative actions of 1,25-$(OH)_2D_3$ and 9-cis RA on LNCaP cells.

Dose response effect of 1,25-$(OH)_2D_3$ on AR mRNA

The effect of 1,25-$(OH)_2D_3$ on steady-state AR mRNA levels was assessed by Northern blot analysis. We have used two culture conditions (FBS medium and CSS me-

dium) in this set of experiments and have observed similar results. As shown in Fig. 1, LNCaP cells express a major transcript of AR at 11 kb. In Fig. 1A, the cells were treated in CSS medium for 24 h with increasing concentrations of 1,25-$(OH)_2D_3$ (0–100 nM), and AR mRNA transcripts increased in a dose-dependent manner. The increased AR mRNA levels became evident with a concentration of 1,25-$(OH)_2D_3$ at 1 nM (lane 3). Increasing the 1,25-$(OH)_2D_3$ concentration caused further induction of AR mRNA (lanes 4–5). The levels of AR mRNA were quantitatively determined by densitometric scanning of the autoradiographs, with correction for the L7 mRNA signal (Fig. 1B). At 100 nM of 1,25-$(OH)_2D_3$, more than 5-fold up-regulation of AR mRNA was detected (lane 5). When we carried out the experiment using FBS medium (Figs. 1, C and D), we also detected a significant up-regulation of AR mRNA in LNCaP cells in response to 1,25-$(OH)_2D_3$ treatment for 24 h. Hence, 1,25-$(OH)_2D_3$ increased AR mRNA expression in LNCaP cells in a dose-dependent manner in either CSS medium or FBS medium.

Time-course of AR mRNA expression in response to 1,25-$(OH)_2D_3$

In Fig. 2, time-course experiments using CSS medium revealed that addition of 10 nM 1,25-$(OH)_2D_3$ to LNCaP cells increased AR mRNA levels by 8 h. The AR mRNA levels peaked at 48 h in the treated cells, with a 10-fold higher level, compared with the untreated cells at the concurrent time point, and this inductive effect of 1,25-$(OH)_2D_3$ was sustained at 72 h. No change in AR mRNA could be detected at 4 h, suggesting a delayed primary response of AR gene expression to 1,25-$(OH)_2D_3$.

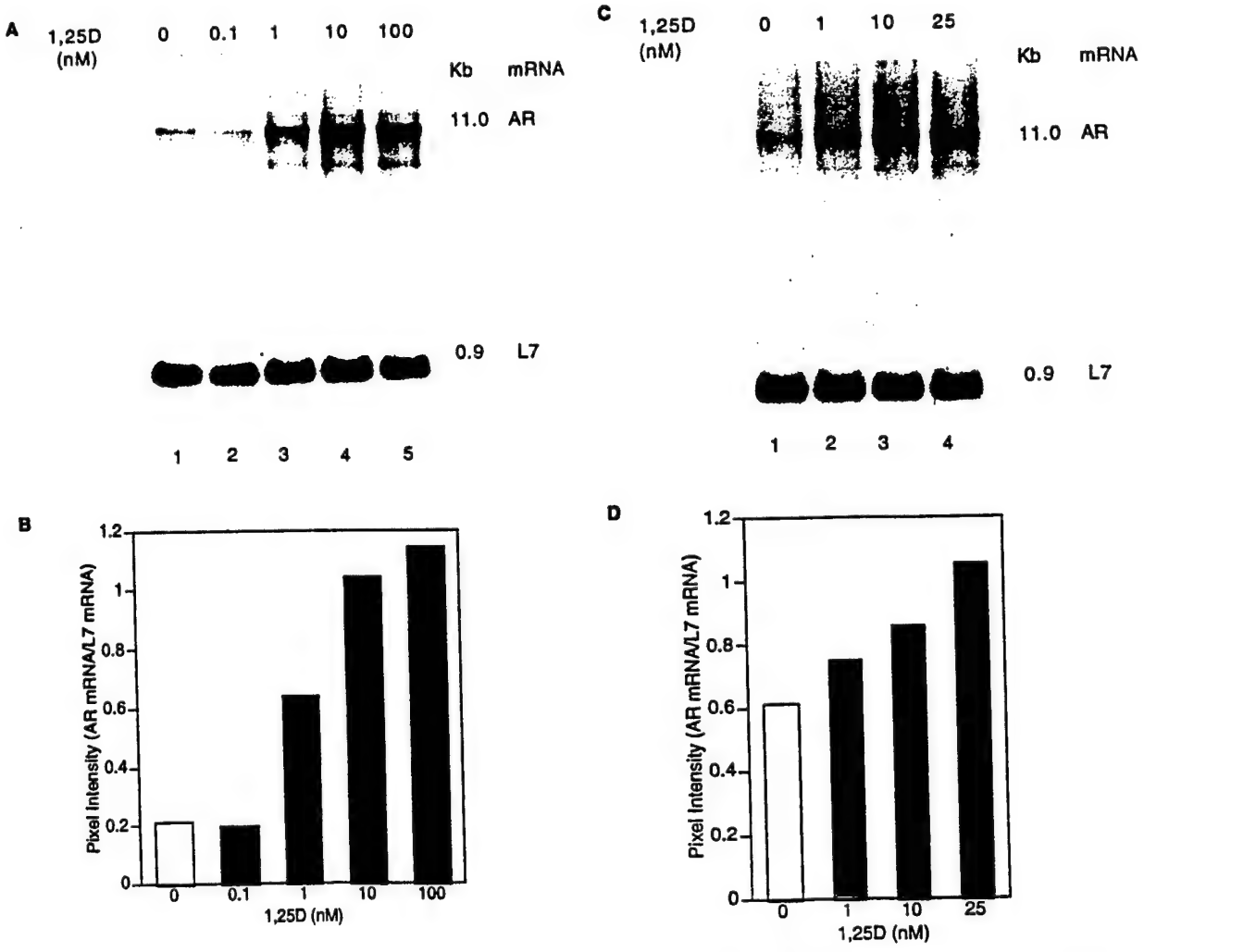

FIG. 1. Dose-dependent effect of 1,25-$(OH)_2D_3$ on AR mRNA levels in LNCaP cells. A, Northern blot analysis in CSS medium. LNCaP cells were treated with 1,25-$(OH)_2D_3$, at the indicated concentrations, for 24 h in RPMI medium containing 5% charcoal stripped serum. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P-labeled 712-bp *Hin*dIII-*Eco*RI fragment of the human AR cDNA at 60 C. The blot was simultaneously probed for expression of the L7 ribosomal protein gene as a control for sample loading and transfer. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA. C, Northern blot analysis in FBS medium. LNCaP cells were treated with 1,25-$(OH)_2D_3$, at the indicated concentrations, for 24 h in RPMI medium containing 5% FBS. Total RNA was isolated, and the RNA blot was hybridized with a ^{32}P-labeled human AR cDNA and the L7 gene. D, The pixel intensity of each AR band indexed to L7 in panel C.

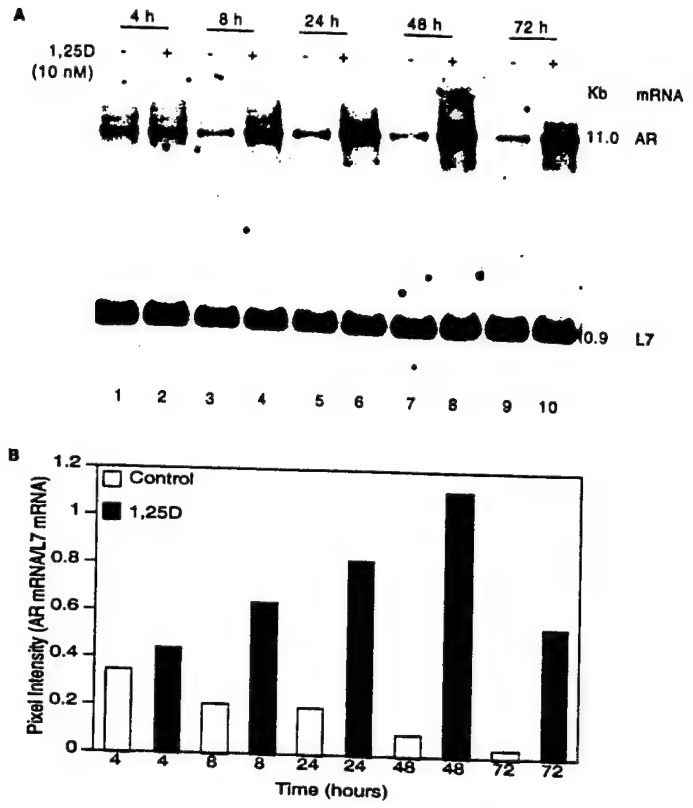

FIG. 2. Time-course of AR mRNA expression in LNCaP cells, in response to 1,25-$(OH)_2D_3$. A, Northern blot analysis. LNCaP cells were treated with 1,25-$(OH)_2D_3$, at 10 nM, for the indicated time period. Total RNA was isolated and analyzed by Northern blot using the human AR cDNA and L7 cDNA as probes. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

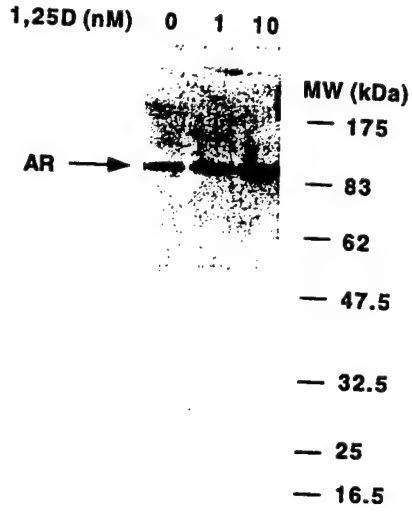

FIG. 3. Western blot analysis of AR protein in LNCaP cells. LNCaP cells were incubated, in RPMI medium containing 5% charcoal stripped serum, with the indicated dose of 1,25-$(OH)_2D_3$ for 2 days. High-salt protein extracts were electrophoresed in an 8% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with anti-AR monoclonal antibody F39.4. Immunoreactive bands were detected by incubation of blots with a secondary antibody (rabbit antimouse IgG), followed by ECL. Molecular weight standards are indicated. hAR is indicated by an *arrow*. The experiment was repeated twice, with similar results.

Up-regulation of the AR protein by 1,25-$(OH)_2D_3$

1,25-$(OH)_2D_3$ also caused a concentration-dependent stimulation of AR protein expression in LNCaP cells cultured in CSS medium, as measured by Western blot analysis (Fig. 3). The major species of AR in LNCaP cells was detected as a single band, at 108 kDa, by monoclonal antibody F39.4. There was no detectable increase in AR protein levels after 24 h treatment (data not shown), but levels rose approximately 4-fold in cells treated with 10 nM 1,25-$(OH)_2D_3$ for 48 h. As seen for AR mRNA levels (Fig. 1), a detectable increase in AR protein was evident with 1 nM 1,25-$(OH)_2D_3$.

[^{3}H]DHT-binding analyses demonstrated that 1,25-$(OH)_2D_3$ increased the AR content in LNCaP cells when they were cultured in either CSS medium or FBS medium. As we have reported (21), cells treated with 1 nM 1,25-$(OH)_2D_3$ in CSS medium showed a more than 2-fold increase in DHT-binding (from 197 ± 17.4 to 430 ± 9.6 fmol/mg protein, n = 3). Addition of 10 nM of 1,25-$(OH)_2D_3$ further up-regulated the AR content (from 197 ± 17.4 to 532 ± 60 fmol/mg, n = 3). Meanwhile, cells cultured in FBS medium exhibited a higher baseline DHT-binding than in CSS medium (378 ± 33.9 fmol/mg *vs.* 197 ± 17.4 fmol/mg, n = 3). 1,25-$(OH)_2D_3$ at 1 nM in FBS medium also increased the AR content (from 378 ± 33.9 to 451 ± 54 fmol/mg protein, n = 3). Therefore, 1,25-$(OH)_2D_3$ up-regulates the AR content in LNCaP cells in either growth condition. Interestingly, CSS medium allowed us to detect a clear up-regulation of AR because charcoal treatment removes endogenous steroids in serum that may interfere with [^{3}H]DHT-binding.

Requirement of new protein synthesis for 1,25-$(OH)_2D_3$ regulation of AR

To determine whether 1,25-$(OH)_2D_3$ affected AR mRNA levels via a direct mechanism, LNCaP cells in CSS medium were treated for 24 h with 1,25-$(OH)_2D_3$ in the presence of the protein synthesis inhibitor cycloheximide (CHX) at various doses (0, 2, 5, and 10 μg/ml). As shown in Fig. 4, CHX blocked the 1,25-$(OH)_2D_3$-induced increase in AR mRNA levels, such that in the presence of CHX and 1,25-$(OH)_2D_3$ (lane 3), AR mRNA levels were no higher than those in untreated cells (lane 1). The extent of blockade depended upon the concentration of CHX included in the media (lanes 3–5). Moreover, the effect of CHX could be detected at either 16 h (lane 6) or 24 h (lane 4).

In other studies, using a 6-kb AR promoter-luciferase reporter transfected into LNCaP cells, we attempted to directly induce expression of AR with 1,25-$(OH)_2D_3$. No increase in luciferase could be detected with 1,25-$(OH)_2D_3$, whereas dibutyrl cAMP induced a 5-fold rise in luciferase (data not shown). Taken together, these findings indicate that 1,25-$(OH)_2D_3$ regulates AR mRNA expression via an indirect mechanism requiring new protein synthesis.

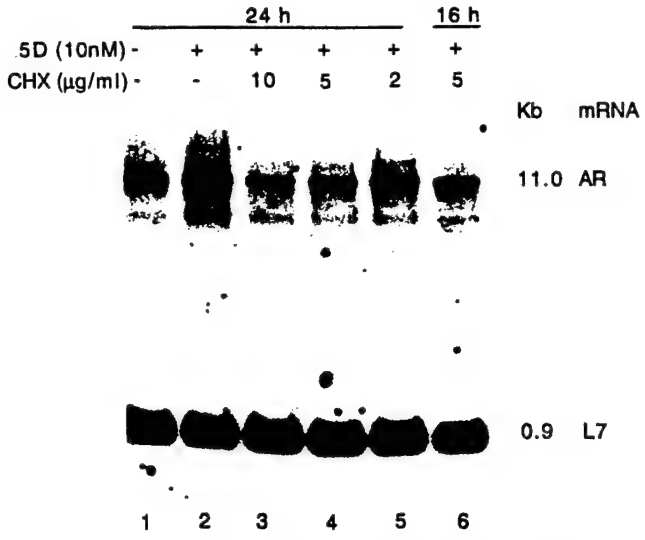

FIG. 4. Effect of CHX on the 1,25-$(OH)_2D_3$ induction of AR mRNA in LNCaP cells. Cells were treated with ethanol (lane 1) or 10 nM 1,25-$(OH)_2D_3$ (lanes 2–6), in the presence of CHX, at the indicated concentrations, for 24 h (lanes 1–5) or 16 h (lane 6). Total RNA was isolated and analyzed by Northern blot using the human AR cDNA and L7 cDNA as probes.

Enhancement of 1,25-$(OH)_2D_3$-mediated up-regulation of AR by 9-cis RA

It recently has been reported that 1,25-$(OH)_2D_3$ acts synergistically with 9-cis RA to inhibit LNCaP cell proliferation [7]. We therefore investigated the effect of 9-cis RA on 1,25-$(OH)_2D_3$ regulation of AR mRNA. Both culture conditions (FBS medium and CSS medium) gave similar results. As shown in Fig. 5, 1,25-$(OH)_2D_3$, at a dose of 10 nM, induced a 5-fold increase in AR mRNA levels (lane 1) over the control at 24 h (lane 2). LNCaP cells, treated with 100 nM 9-cis RA for 24 h, expressed 3-fold more AR mRNA (lane 3) than the untreated cells (lane 2). Combination treatment of 1,25-$(OH)_2D_3$ and 9-cis RA gave a more than 8-fold induction of AR mRNA (lane 4). Thus, although 1,25-$(OH)_2D_3$ was more effective than 9-cis RA in up-regulating AR mRNA, both hormones acted additively to increase AR gene expression in LNCaP cells.

FIG. 5. Enhancement of 1,25-$(OH)_2D_3$ action by 9-cis RA in the induction of AR mRNA in LNCaP cells. A, Northern blot analysis. LNCaP cells were treated with 1,25-$(OH)_2D_3$ at 10 nM, or 9-cis RA at 100 nM, individually or in combination for 24 h. Total RNA was extracted and analyzed by Northern blot. B, The pixel intensity of each AR band in panel A was scanned by computing densitometer, and the data were integrated by scanner software and indexed to the corresponding levels of L7 mRNA.

Involvement of AR action in the antiproliferative response of 1,25-$(OH)_2D_3$ and 9-cis RA

We further tested the possible involvement of AR action in the antiproliferative effect of 1,25-$(OH)_2D_3$ and its synergism with 9-cis RA. As shown in Fig. 6A, 10 nM 1,25-$(OH)_2D_3$ inhibited LNCaP cell growth 50%, whereas 100 nM 9-cis RA only reduced proliferation by 10%. However, combination treatment with 1,25-$(OH)_2D_3$ and 9-cis RA caused 80% growth inhibition. Casodex was used to determine whether the 9-cis RA action was also AR-dependent. In the presence of Casodex, neither 1,25-$(OH)_2D_3$ nor 9-cis RA inhibited cell growth individually or in combination (Fig. 6A). On the other hand, dibutyrl cAMP, a known up-regulator of AR in LNCaP cells, inhibited cell proliferation in a dose-dependent manner (Fig. 6B). With maximal growth inhibition of 90% occurring at a dose of 5 mM dibutyrl cAMP, addition of Casodex, however, did not block this effect (Fig. 6B). These data imply that the actions of 1,25-$(OH)_2D_3$ and 9-cis RA on LNCaP cell growth are both AR-dependent. In contrast, cAMP, although an inducer of AR (23), inhibits LNCaP cell proliferation by an AR-independent mechanism.

Model of 1,25-$(OH)_2D_3$ and 9-cis RA action in LNCaP

The hormonal action of 1,25-$(OH)_2D_3$ is mediated by the VDR present in LNCaP cells. Figure 7 depicts the possible events in the 1,25-$(OH)_2D_3$ signaling pathway in these cells. 1,25-$(OH)_2D_3$ binds to the VDR and activates the receptor. The activated VDR controls target gene transcription by forming a heterodimer with the partner RXR and binding to the VDRE in the promoter region of a target gene. A 1,25-$(OH)_2D_3$ target gene (or genes) encodes protein(s) X, mediators of the up-regulation of AR mRNA in response to 1,25-$(OH)_2D_3$. The production of protein(s) X is CHX-sensitive. Both 1,25-$(OH)_2D_3$ and 9-cis RA, individually or in combi-

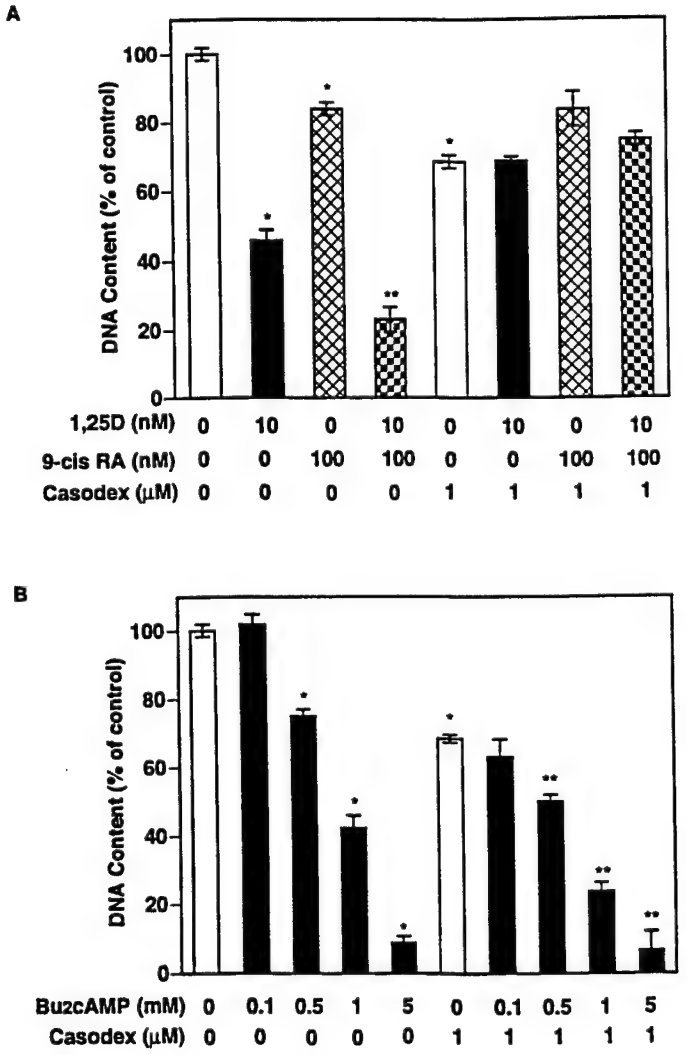

FIG. 6. Effect of Casodex on 1,25-$(OH)_2D_3$ and 9-cis RA-induced growth inhibition on LNCaP cells. A, LNCaP cells were treated with ethanol, 1,25-$(OH)_2D_3$ at 10 nM, or 9-cis RA at 100 nM, individually or both in the presence or absence of Casodex at 1 μM, for 6 days. Cellular DNA contents were determined by Burton's method. The data are expressed as percent of control, a mean of three triplicate samples ± SEM. *, $P < 0.01$, compared with the untreated control group; **, $P < 0.05$, compared with the single-treatment group. B, LNCaP cells were treated with dibutyrl cAMP [Bu2cAMP], from 0–5 mM, in the presence or absence of Casodex at 1 μM, for 6 days.

nation, induce AR mRNA expression. The increased AR mRNA causes an increase in AR protein levels, which mediates the action of androgens on LNCaP cell growth. As we reported previously (23), either 1,25-$(OH)_2D_3$ or DHT was growth inhibitory on LNCaP when cells were cultured in FBS-containing medium. The androgen action is blocked by the AR antagonist, Casodex. Although both the AR mRNA and the AR protein are also induced in CSS medium, in the absence of androgens, 1,25-$(OH)_2D_3$ does not exhibit an antiproliferative effect on LNCaP cells in this culture system (21).

Discussion

The AR is the key element in the androgen signal transduction cascade, and it plays a critical role in the regulation of growth and differentiation of the prostate. The data presented here demonstrate that 1,25-$(OH)_2D_3$ up-regulates AR gene expression at both mRNA and protein levels in LNCaP cells, an androgen-responsive human prostate cancer cell line. This inductive action of 1,25-$(OH)_2D_3$ was enhanced by 9-cis RA, which by itself also up-regulates AR expression in LNCaP cells. Our data show that growth inhibition induced by 1,25-$(OH)_2D_3$ alone or in combination with 9-cis RA was accompanied by increased AR expression. Moreover, the antiproliferative actions of 1,25-$(OH)_2D_3$ and 9-cis RA were AR-dependent and could be blocked by the AR antagonist, Casodex. Although androgens are not added in these experiments, androgens are present in the serum supplement to the culture medium (FBS medium), and we hypothesize that the amplitude of the androgen response is augmented by the increased levels of AR expressed in these cells after treatment with 1,25-$(OH)_2D_3$ and/or 9-cis RA. Support for the effect of androgens, in FBS medium, on cell growth was presented in our earlier studies (21). In the absence of androgens (for example, in CSS medium), 1,25-$(OH)_2D_3$ does not exhibit an antiproliferative action on LNCaP cells.

Nonetheless, our finding that androgen mediates the antiproliferative activity of 1,25-$(OH)_2D_3$ in LNCaP cells is not the situation in all prostate cancer cells. 1,25-$(OH)_2D_3$ inhibits the growth of AR-negative prostate cancer cell line PC-3, as well as primary cultures of human prostate cells. In contrast to LNCaP cells, mechanisms other than androgen signaling are responsible for the growth inhibitory effect of 1,25-$(OH)_2D_3$ on these cells.

It is of interest to consider whether increasing the abundance of a steroid receptor, such as the AR, will cause an increased amplitude of response, *i.e.* antiproliferation. Although it has been well demonstrated that the level of receptors in LNCaP does not necessarily predict the ligand potency of a hormonal response (8, 14, 19, 27), it is clear that the presence of a receptor is essential for a response (1, 2, 13, 14, 28). In a given cell, in the presence of a constant level of hormone, up-regulation of the receptor does cause an enhanced response, whereas down-regulation of the receptor diminishes the response (13, 14, 28–30). Therefore, we believe that up-regulation of AR, in these studies, is the mechanism of the enhanced antiproliferative effect.

The expression of the AR gene has been found to be induced by a number of agents in several systems, such as the rat ventral prostate (31), and in LNCaP human prostate cancer cells (32, 33). Growth factors such as FSH, EGF, and TGF-β regulate AR gene expression (34, 35). Activators of protein kinase A, such as forskolin and dibutyrl cAMP, are the known up-regulators of AR in LNCaP cells (23). The induction of AR by these reagents was not detected in the two other commonly studied prostate cancer cell lines, PC-3 and DU 145, which do not express basal levels of AR mRNA (36).

1,25-$(OH)_2D_3$ is the most potent inducer of AR in LNCaP cells, among the three agents that we tested. Consistent with the reported data (23), we found that treatment of LNCaP cells with dibutyrl cAMP for 24 h caused a 2-fold increase in AR mRNA levels (data not shown). In the same experiment, we observed an increase of 5-fold in AR mRNA, with 1,25-$(OH)_2D_3$ at 10 nM. 9-cis RA induced 3-fold induction of AR mRNA. It has been reported that dibutyrl cAMP increases

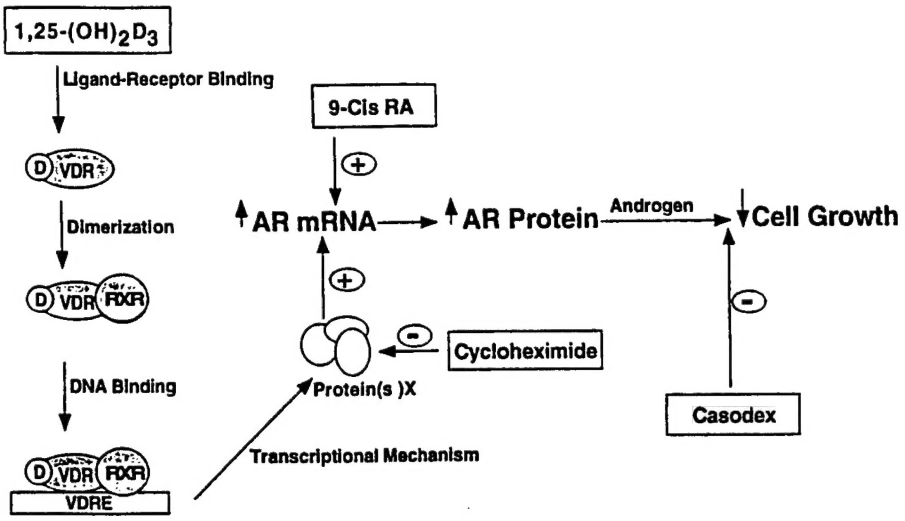

FIG. 7. A tentative model of 1,25-$(OH)_2D_3$ and 9-cis RA action on LNCaP cells. Both 1,25-$(OH)_2D_3$ and 9-cis RA induce AR mRNA expression. The increased AR mRNA leads to an increase in AR protein levels. AR protein mediates androgen action in cell proliferation. The pure AR antagonist, Casodex, blocks AR action; in turn, it blocks the growth-inhibitory action of 1,25-$(OH)_2D_3$ and 9-cis RA.

AR gene transcription via the cAMP-response elements present in the 2.3-kb promoter region of the human AR gene (23). In contrast, the same promoter region of the AR gene seems to lack a VDRE and an RXRE. Computer searching of the 2.3-kb promoter region failed to identify a consensus sequence for these regulatory elements. Moreover, the luciferase reporter construct, driven by a 6-kb promoter region of the human AR gene, did not respond to 1,25-$(OH)_2D_3$ or 9-cis RA but did respond to dibutyrl cAMP.

An indirect mechanism for 1,25-$(OH)_2D_3$ action to induce AR was supported by several findings taken together: the delayed time of AR mRNA rise in time-course experiments (Fig. 2), the CHX studies (Fig. 3), and the failure of the promoter to respond to 1,25-$(OH)_2D_3$ (data not shown). We refer to the indirect, CHX-inhibited mediator(s) of 1,25-$(OH)_2D_3$ action to up-regulate AR as protein(s) X. We surmise that, in the presence of CHX, 1,25-$(OH)_2D_3$ was unable to induce protein(s) X, and as a consequence, 1,25-$(OH)_2D_3$ failed to up-regulate AR mRNA (Fig. 4). It is interesting to speculate on the nature of protein(s) X. Protein(s) X may be related to the chaperon proteins, given the fact that several chaperons have been identified in the regulation of steroid receptors (37). Further studies are needed to elucidate this mechanism.

We did not detect AR up-regulation by 1,25-$(OH)_2D_3$ in two human breast cancer cells, either MCF-7 or T47D (unpublished data). Both MCF-7 and T47D cells express the VDR, as well as the AR. However, the levels of AR protein did not change in both cell lines when treated with 1,25-$(OH)_2D_3$. Therefore, induction of protein(s) X by 1,25-$(OH)_2D_3$ may be tissue-specific. At present, it is difficult to examine this point because of the limited number of human prostate cancer cells that exhibit the AR. We have, thus far, been unable to induce AR in cells that lack the AR, including primary cultures of prostate cancer cells and established cell lines PC-3 or DU 145. To determine whether AR induction by 1,25-$(OH)_2D_3$ is LNCaP cell-specific, we hope to study other AR-positive human prostate cancer cell lines as they become available.

The action of androgens to inhibit proliferation of cultured prostate cancer cells is an interesting finding. We and others (21, 38) showed that LNCaP cells exhibit a biphasic growth response to DHT in charcoal-stripped serum-containing medium, with a growth stimulatory effect at a low concentration (less than 1 nM) and an inhibitory effect at a high concentration (greater than 1 nM). The levels of AR protein in LNCaP cells determine the concentration of DHT at which the stimulatory effect crosses over to an inhibitory effect. In other words, the stimulatory effect is favored at low abundance of AR, and an inhibitory effect at high abundance of AR (21). Liao and co-workers (39–41) found that high-passage LNCaP cells in an androgen-depleted medium express 10- to 20-fold higher AR levels and are growth inhibited by androgens *in vitro* and in an *in vivo* mouse model. Moreover, they demonstrated that G1 arrest of the high AR-expressing cells by androgen is caused by the induction of $p27^{kip1}$, which in turn inhibits Cdk2, a factor critical for cell cycle progression and proliferation (41). There are two additional examples to document the role of AR in the inhibition of growth of prostate cancer cells. Yuan *et al.* (42) have reported that PC-3 cells, stably transfected with the human AR cDNA, were growth inhibited by androgen. Recently, Zhau *et al.* (43) have established an androgen-repressed human prostate cancer cell line (ARCaP) derived from the ascites fluid of a patient with advanced metastatic disease, which is growth inhibited by androgens. Cumulatively, these findings support the hypothesis that higher levels of AR in cultured prostate cancer cells cause increased sensitivity to growth inhibition.

In summary, we have shown that the hormonally active forms of vitamin D and vitamin A are potent inducers of AR in LNCaP cells. Both 1,25-$(OH)_2D_3$ and 9-cis RA act in synergy to inhibit cell proliferation; moreover, their antiproliferative actions can be blocked by the AR antagonist, Casodex. In conclusion, our study provides direct evidence for an important role of the AR in mediating the growth inhibitory actions of 1,25-$(OH)_2D_3$ and 9-cis RA in LNCaP cells. More importantly, the newly discovered AR-inducing property of both vitamins A and D suggests a possible application of these potential chemo-preventive agents in increasing androgen sensitivity of prostate cancer cells. An understanding of the mechanisms of AR gene

regulation may be of great importance in efforts to restore androgen responsiveness to the patients with androgen-independent prostate cancer, because this type of cancer is commonly unresponsive to most conventional therapies.

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factors and cell adhesion molecules, many of which also contribute to prostate carcinogenesis, have been identified. Moreover, it is now accepted that the prostate is an endocrine gland capable to synthesize and secrete a wide variety of hormones. It has been reported that gonadotropins are expressed in the human prostate (Br J Cancer 1990, 61:225). In this study we analized by reverse transcriptase-polimerase chain reaction (RT-PCR) the mRNA expression for FSH receptor in human prostate. Prostate tissue was obtained from a total of 18 patients (age 55-79 years). Each sample was evaluated histologically. Four patients were affected by benign prostatic hyperplasia (BPH) and 14 by adenocarcinoma. Specimens of normal tissue were obtained in four patients with carcinoma from the portion of the gland not affected by the tumor. The samples were quickly frozen and kept at -80 °C. Total RNA was extracted and RT-PCR performed on equal amounts of RNA samples. Specific primers for exon 10 of the FSH receptor gene were used. The reaction was carried out in 50 μl, after an initial denaturation for 3 min at 94 °C, for 40 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min), followed by a final elongation of 5 min at 72 °C. Control PCR reactions were performed on RNA samples before reverse transcription to confirm the absence of genomic DNA contamination. PCR products were resolved and visualized on 1.5% agarose gels stained by ethidium bromide. The results obtained show that the FSH receptor mRNA is expressed in human prostate. All the normal tissues examined showed low levels of expression. Two out of four BPH and eight out of fourtheen adenocarcinoma were positive, showing higher levels of expression of the gene compared to normal, with a wide degree of variability between the samples. No correlation with histological Gleason grading of the adenocarcinoma specimens was found. The expression of the FSH receptor in human prostate and its up regulation in some cases of BPH and carcinoma suggests that FSH may play a role in the development of some prostatic disorders, as a factor that takes over androgen regulation of prostatic cell proliferation during the development of androgen-independent growth. Moreover, the recently reported down regulation of the inhibin-α gene expression in prostate cancer (J Clin Endocrinol Metab 1998, 83:969) supports the concept of an autocrine/paracrine regulation of FSH action within the prostate.

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DIFFERENT EXPRESSION OF ESTROGEN RECEPTOR α AND β IN PRIMARY CULTURES OF NORMAL AND MALIGNANT HUMAN PROSTATE EPITHELIAL CELLS.

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The estrogen receptor α (ERα) has been localized to the stroma and basal epithelial cells of human prostate. Recently an high level of expression of a novel ER subtype, ERβ, has been demonstrated in rat prostate epithelium, but the presence and the role of this subtype in human prostate is unknown. In this study we investigated the expression ER α and β genes and screened the presence of receptor proteins in normal (N) and malignant (K) prostate epithelial cell (EC) primary cultures. ***Methods:*** Primary epithelial cell cultures were established in SFM-keratinocyte medium (Gibco-BRL) with growth factors and 5% FCS, after collagenase digestion of minced N and K prostate surgical samples. mRNA transcripts of ER α and β were detected by RT-PCR using specific primers amplifying a region comprise between exon 4 and 6 (set 1). ER a expression was further studied using a set of primers comprised between exon 6 and exon 8 (set 2). Receptor proteins were searched in cell lysate by by Western blotting (WB) using a monoclonal antibody raised against a C-terminal epitopes common to ER α and β, and another raised against N-terminal part of ER α. ***Results:*** In NEC both ER α and β genes were expressed. In KEC only ER α mRNA was clearly detected when amplified with set 1. By WB, using the C-terminus directed antibody, the ER α and β proteins were found in cell lysates from NEC, while resulted absent in that from KEC cultures. Using an antibody directed against the N-terminus domain of ERα, the protein was found both in NEC and KEC. ***Conclusions:*** We demonstrated the presence of ER α and β gene transcripts and relative proteins in cultured EC from normal human prostates. In KEC ERβ mRNA and protein were undetectable, and ER α gene resulted translated in a protein probably changed in C-terminal domain.

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INHIBITION OF HUMAN PROSTATE CANCER CELLS GROWTH BY ANTIOXIDANTS IS MEDIATED THROUGH APOPTOSIS.

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Introduction. Many human prostate cancer cells have escaped the apoptotic effects of natural regulators of cell growth such as transforming growth factor β1 (TGFβ-1) and tumor necrosis factor. Some evidence suggests that antioxidants might suppress the development of prostate cancer.

Methods. To investigate if prostate cancer cell growth can be influenced by apoptotic activators, DU 145 (androgen unresponsive), ALVA 101 (moderately androgen responsive) and LNCaP (androgen responsive) were grown in RPMI 1640 medium supplemented with bovine calf serum and antibiotics and were treated with various antioxidants for 1-7 days. Cell growth was then determined with the Cell Titer 96 AQ assay (Promega). Apoptosis was assessed by cell death detection ELISA assay (quantitating histone-associated DNA fragments) in the cytoplasm of cells, and cell viability by methylene blue exclusion.

Results. (±)-α-Tocopherol (vitamin E) treatment for 1-7 days at concentrations of 0.08-5 μg/mL modestly affected growth compared to other antioxidants tested. Tocopherol produced a significant ($p<0.01$) inhibition of ALVA 101 and LNCaP (10% inhibition compared to control; 2.5 μg/mL; at 6-7 days; n=6), but did not show a dose response inhibition. DU 145 cells were not growth inhibited with tocopherol treatment. However, pyrrolidinedithiocarbamate (PDTC) produced a significant ($p<0.01$, n=6) dose response inhibition (20-80% inhibition) of DU 145 and ALVA 101 cells and a significant ($p<0.01$) inhibition of LNCaP cells without a dose response relationship also at concentrations of 2.5-20 μg/mL. When cells were grown to confluence, PDTC failed to inhibit cell growth. A third compound, diethyldithiocarbamic acid (DETC), incubated for 1-7 days also produced significant dose response suppression of cell growth of DU 145 and ALVA 101 cells ($p<0.01$; 20-80% inhibition; n=6) at concentrations of 1.25-80 μg/mL. LNCaP cells were inhibited ($p<0.01$; 28% inhibition; n=6) by DETC, but they did not show a dose response inhibition compared to the other two cell lines. ELISA assay of nucleosomes obtained from ALVA 101 cells treated with PDTC documented significant ($p<0.03$; n=6) apoptosis compared to untreated controls.

Conclusions. These results demonstrate that antioxidants modulate human prostate cancer cell proliferation by altering apoptosis, but they do not cause necrosis or apoptosis of confluent cells.

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ANDROGEN INSENSITIVITY DUE TO A DOUBLE MUTATION IN THE ANDROGEN RECEPTOR OF A NEW HUMAN PROSTATE CANCER CELL LINE MDA PCa 2a.

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We have characterized the androgen receptor (AR) in a new human prostate cancer cell line, MDA PCa 2a, that has recently been established from a bone metastasis of a patient whose cancer was exhibiting androgen-independent growth. These cells express abundant AR (Nmax=685±149 fmol/mg protein), as determined by equilibrium binding assays with [3H]DHT. However, Scatchard analyses showed the AR binding affinity for DHT in these cells to be only 25 nM, 50-fold lower than the AR in LNCaP cells (Kd=0.5 nM) or the wildtype AR in MCF-7 cells (Kd=0.4 nM). DNA sequencing analyses of the AR gene in MDA PCa 2a cells revealed two mutations in the ligand-binding domain, L701H and T877A, the latter being reported previously in the LNCaP cells. Compared to LNCaP, the new cell line is 10 to 1000-fold less responsive to androgens in cell growth assays as well as in stimulation of PSA. Interestingly, in the absence of added androgens, the new cell line expresses 15-fold higher baseline levels of PSA than LNCaP, suggesting constitutive expression of its PSA protein. We are currently recreating both mutations by *in vitro* mutagenesis and evaluating the recreated mutant AR in terms of its ligand binding affinity, specificity, and transactivation properties. In summary, we have identified two mutations in the AR gene of MDA PCa 2a cell line that are likely responsible for the decreased binding affinity for DHT and the partial androgen insensitivity observed in these cells. Thus, this new cell line can serve as a functionally relevant model system of advanced prostate cancer with bone metastases.

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SOMATOSTATIN AFFECTS THE LEVELS OF TOPOISOMERASE II GENE AND PROTEIN IN LNCaP CELLS.

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Somatostatin (S) has been previously shown to exert an inhibitory effect on the growth of the human prostatic cancer cell line LNCaP (Lymph Node Carcinoma of the Prostate). However, the molecular mechanisms, possibly involved in the hormone antiproliferative action remain still elusive and have to be further elucidated. We recently reported the effect of S on LNCaP cell gene transcription, using RNA differential display (dd)PCR techniques. In particular we identified up-regulated levels of expression of a 170 cDNA fragment in LNCaP cells exposed to S. The interaction of the hormone with the present transcript was futher confirmed by both Northern Analysis and Ribonuclease Protection Assay. Comparison of its cDNA sequence with the Genebank and EMBL databases revealed a 91% degree of homology with the deposited sequence of the nuclear enzyme Topoisomerase II. To investigate the physiological impact of this finding we evaluated the effect of the hormone on the corresponding protein. To this purpose we cultured LNCaP cells either in the presence or in the absence of S. We then extracted LNCaP cell constitutive proteins and separated them on a 8-15% SDS PAGE. Proteins were blotted onto Nitrocellulose filters and probed for the levels of Topoisomerase II protein using a specific monoclonal antibody. The results obtained indicate that S interacts with both the levels of transcription and the concentration of Topoisomerase II protein when used at the same dose shown to exert an antiproliferative effect on LNCaP cells.

This enzyme is a key molecule regulating cell cycle and, more specifically, chromosome condensation and mitosis. Its levels as well as its timing of expression are considered to be crucial for the correct completion of cell cycle. Thus, alteration of these parametres may lead to an uncoordinated replication cycle.

The observation that S probably enhances the levels of Topoisomerase II molecule suggests the possibility that the hormone might affect LNCaP cell proliferation directly interferring with this enzyme and, therefore, with the correct cell cycle dynamic in LNCaP cell line.

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mechanism of action of AA, the effect of AA on PKC activity was examined by adding inhibitors of PKA (H8 and H89), lipoxygenase (NDGA and esculetin), or cyclooxygenase (indomethacin) to the cultures in the presence or absence of 24,25. The identification of which PKC isoform is activated by AA was determined by PKC isoform-specific antibodies. AA stimulated [^{3}H]-thymidine incorporation and inhibited the activity of alkaline phosphatase and PKC, but had no effect on matrix production. In contrast, 24,25 inhibited [^{3}H]-thymidine incorporation and stimulated alkaline phosphatase and PKC as well as matrix production. In cultures treated with both agents, the effects of 24,25 were abrogated by AA. Studies using cyclooxygenase and lipoxygenase inhibitors indicated that the effects of AA were due in part to prostaglandins but not leukotrienes. AA did not alter the translocation of PKC from the cytosol to the membrane. Direct addition of AA to isolated matrix vesicles activated PKC, while addition to plasma membranes inhibited PKC. This was opposite to the effect seen with 24,25; in addition, when added to the membranes at the same time, AA abrogated the effect of 24,25. Studies with PKC isoform-specific antibodies indicated that the predominant PKC isoform affected by AA treatment was PKCα. This study shows that AA regulates RC proliferation, differentiation, and matrix production. The effect of AA on the cultures was the opposite of that observed with 24,25. We conclude that 24,25 mediates its effects on RC cells by inhibiting AA production. AA also had a direct effect on membranes isolated from the cultures, suggesting that there may be a unique membrane receptor for AA.

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Liarozole Acts Synergistically With 1,25-Dihydroxyvitamin D3 to Inhibit DU 145 Human Prostate Cancer Cell Growth by Blocking 24-Hydroxylase Activity. X. Y. Zhao, Lan H. Ly, Leah Holloway, David Feldman. Department of Medicine, Stanford University, Stanford, CA.

1α,25-Dihydroxyvitamin D3 [1,25-(OH)2D3] inhibits the proliferation of many cancer cells in culture but not the aggressive human prostate cancer cell line DU 145. We postulated that the 1,25-(OH)2D3-resistant phenotype of DU 145 cells might result from the high levels of expression of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) induced by treatment with 1,25-(OH)2D3. Since this P450 enzyme initiates 1,25-(OH)2D3 catabolism, we presumed a high level of enzyme induction could limit the effectiveness of the 1,25-(OH)2D3 antiproliferative action. To examine this hypothesis we explored combination therapy with liarozole fumarate (R85,246), an imidazole derivative currently in trials for prostate cancer therapy. Since imidizole derivatives are known to inhibit P450 enzymes, we postulated that this drug would inhibit 24-hydroxylase activity, increasing 1,25-(OH)2D3 half-life, thereby enhancing 1,25-(OH)2D3 antiproliferative effects on DU 145 cells. Cell growth was assessed by measurement of viable cells using the MTS assay. Neither 1,25-(OH)2D3 (1-10 nM) nor liarozole (1-10 μM) inhibited DU 145 cell growth when given alone. However, together at 4 days, 1,25-(OH)2D3 (10 nM)/liarozole (1 μM) inhibited growth 65%. We used a thin layer chromatography method to assess 24-hydroxylase activity and demonstrated that liarozole (1-100 μM) inhibited this P450 enzyme in a dose-dependent manner. Moreover, liarozole treatment caused a significant increase in 1,25-(OH)2D3 half-life from 11 to 31 h. In addition, 1,25-(OH)2D3 can cause homologous up-regulation of the VDR and in the presence of liarozole, this effect was augmented thus enhancing 1,25-(OH)2D3 activity. Western blot analyses demonstrated that DU 145 cells treated with 1,25-(OH)2D3/liarozole showed greater VDR up-regulation than with either drug alone. In summary, our data demonstrate that liarozole augments the ability of 1,25-(OH)2D3 to inhibit DU 145 cell growth. The mechanism appears to be due to inhibition of 24-hydroxylase activity leading to increased 1,25-(OH)2D3 half-life and augmentation of homologous up-regulation of VDR. We raise the possibility that combination therapy using 1,25-(OH)2D3 and liarozole or other inhibitors of 24-hydroxylase, both in non-toxic doses, might serve as an effective treatment for prostate cancer.

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Expression of 11β-Hydroxysteroid Dehydrogenase Type 2 in Rat Osteosarcoma Cells: Autocrine Regulation of Glucocorticoid Responses in Bone. M. Hewison, L. J. Eyre,* R. Bland,* M. C. Sheppard,* P. M. Stewart.* Department of Medicine, The University of Birmingham, Birmingham, UK.

The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) acts as a prereceptor signaling mechanism for corticosteroids, by regulating the access of active glucocorticoids to both glucocorticoid (GR) and mineralocorticoid receptors (MR). To examine a possible relationship between endogenous glucocorticoid metabolism and osteoblast activity, we have characterized the expression of 11β-HSD isozymes in rat osteosarcoma cells. Analysis of mRNA from ROS 25/1, UMR106 and ROS 17/2.8 cells revealed transcripts for both 11β-HSD type 1 (11β-HSD1) and type 2 (11β-HSD2) in all three cell lines. However, enzyme activity studies showed only high affinity (Km = 45 nM) dehydrogenase activity (inactivation of corticosterone to 11-dehydrocorticosterone), characteristic of 11β-HSD2. Conversion of corticosterone to 11-dehydrocorticosterone in ROS 25/1 > UMR106 > ROS 17/2.8 cells. Activity in all three cell lines was up-regulated in an autocrine fashion following treatment with natural glucocorticoids, but not dexamethasone (dex). Induction of 11β-HSD2 was unaffected by co-treatment with the GR antagonist RU486 or the MR antagonist RU752, but was completely inhibited by treatment with 11β-HSD inhibitor glycyrrhetinic acid. Analysis of ^{3}H-dex binding revealed approximately 60,000 GR/cell in all three cell lines, with much lower levels of MR detected using ^{3}H-aldosterone (aldo). Parallel studies of alkaline phosphatase activity indicated that dex and corticosterone stimulated activity in ROS 17/2.8 cells but had no effect on ROS 25/1 cells or UMR106; the classical MR ligand, aldo, was without effect in all three cell lines. Up-regulation of alkaline phosphatase activity in ROS 17/2.8 by dex was completely inhibited by RU486 whereas RU752 had no effect. In contrast, induction of alkaline phosphatase activity by corticosterone was inhibited by both RU486 and RU752, suggesting different receptor mechanisms for osteoblast responses to synthetic versus naturally occurring glucocorticoids. These studies demonstrate capacity for local inactivation of glucocorticoids in osteoblasts. The presence of GR and lack of functional response to aldo suggest that 11β-HSD2 in rat osteoblasts acts in a protective fashion by regulating GR occupancy; cells with relatively high levels of 11β-HSD2 activity (ROS 25/1 and UMR106) were insensitive to glucocorticoids, whilst cells with low levels demonstrated functional responses to both dex and corticosterone. We therefore postulate that pre-receptor regulation of ligand availability is a major determinant of glucocorticoid effects on bone.

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Glucocorticoids Inhibit the Expression of Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in Osteoblasts. Frederic Blanquaert, Ernesto Canalis. Saint Francis Hospital and Medical Center, Hartford, CT.

Glucocorticoids have profound effects on skeletal metabolism, and delay wound and possibly fracture healing. However, the mechanisms involved are not known. Hepatocyte growth factor/scatter factor (HGF/SF) plays a central role in tissue regeneration and healing and enhances the replication of cells of the osteoblastic lineage. Expression of HGF/SF in osteoblasts is limited, but it is enhanced by factors that accelerate fracture repair, confirming the role of HGF/SF in healing. We examined the effects of cortisol on HGF/SF expression in osteoblast enriched cells from 22 day fetal rat calvariae (Ob cells). Cortisol at 1 uM for 24 to 48 h decreased the basal expression of HGF/SF transcripts in Ob cells by approximately 50%. In contrast, fibroblast growth factor (FGF) -2 at 2 nM and platelet derived growth factor (PDGF) BB at 3 nM for 24 h significantly increased HGF/SF transcripts by 2 to 8 fold in osteoblast cultures. Cortisol at 1 uM significantly decreased the stimulatory effect of FGF-2 and PDGF BB on HGF/SF expression in Ob cells. An analogous effect was observed in the MC3T3 osteoblastic cell line. Cortisol also caused a time and dose dependent increase in the level of expression of c-met, a protooncogene encoding for the HGF/SF receptor. This effect was observed at cortisol concentrations of 10 to 1,000 nM and after 6 to 24 h cortisol increased c-met transcripts by 3 fold. In conclusion, FGF-2 and PDGF BB increase HGF/SF mRNA expression, and cortisol decreases basal and growth factor-induced HGF/SF transcripts. This effect may be critical to the inhibitory actions of glucocorticoids on fracture healing. Cortisol induction of c-met mRNA levels may be a compensatory mechanism to maintain HGF/SF function in osteoblasts in conditions of glucocorticoid excess.

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Calreticulin Regulates Integrin $\alpha_v\beta_3$ Response To 1α,25(OH)$_2$D$_3$. Zhijie Chang,[1] K. A. Hruska,[2] S. Dedhar, S. L. Teitelbaum,[2] F. P. Ross,[2] Xu Cao.[1] [1]Department of Pathology, University of Alabama, Birmingham, AL, [2]Washington University, St. Louis, MO.

1α,25(OH)$_2$D$_3$ plays an important role in osteoclast differentiation. Calreticulin, a calcium-binding protein, can activate gene transcription such as $\alpha_v\beta_3$. 1 α,25(OH)$_2$D$_3$ transactivition activity is modulated by calreticulin, which binds to the DNA binding domain of 1 α,25(OH)$_2$D$_3$ receptor.

In this study we demonstrated that calreticulin dose-dependently inhibited both VDR binding and its transactivation by transfection of integrin β_3 and VDRE and by gel-shift. Further more, we showed that over-expression of calreticulin blocks 1 α,25(OH)$_2$D$_3$-induced integrin β_3 mRNA level by Northern blot analysis. Whereas, the over-expression of anti-sense calreticulin cDNA enhanced β_3 mRNA levels in HD11 cells treated with 1 α,25(OH)$_2$D$_3$. To further examine the function of calreticulin in osteoclast differentiation, we constructed calreticulin-adenovirus expression vector which can express calreticulin at very high level in transfected osteoclast like cells. The bone resorption and cell attachment was inhibited in the osteoclast precursors infected with adenovirus bearing calreticulin, compared with cells infected with adenovirus alone. Finally, we use immunostaining assay to demonstrate that calreticulin was involved in VDR translocation into nucleus. In summary, we conclude that calreticulin inhibits integrin β_3 expression by direct interacting with VDR, and affects attachment and bone resorption mediated by osteoclasts.